

**COMPARISON OF SEVERAL PROTOCOLS FOR THE INCREASE IN
HOMOLOGOUS RECOMBINATION IN NORMAL PORCINE FETAL
FIBROBLASTS AND THE APPLICATION TO AN ACTUAL LOCUS**

A Dissertation

by

GRETCHEN MARIE ZAUNBRECHER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Veterinary Anatomy

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Approved as to style and content by:

Jorge A. Piedrahita
(Co-Chair of Committee)

James N. Derr
(Co-Chair of Committee)

Loren C. Skow
(Member)

Fuller W. Bazer
(Member)

Evelyn Tiffany-Castiglioni
Head of Department

May 2004

Major Subject: Veterinary Anatomy

ABSTRACT

Comparison of Several Protocols for the Increase in Homologous Recombination in Normal Porcine Fetal Fibroblasts and the Application to an Actual Locus. (May 2004)

Gretchen Marie Zaunbrecher, B.S., University of Southwestern Louisiana;

M.S., Texas Tech University

Co-Chairs of Advisory Committee: Dr. Jorge A. Piedrahita
Dr. James N. Derr

Together with the advancements in animal cloning, the ability to efficiently target specific genes in somatic cells would greatly enhance several areas of research. While it has been possible for quite some time to target specific genes in the germ cells of mice, the advancements in somatic cell gene targeting has been slowed for two main reasons. First, the finite lifespan of somatic cells, due mainly to the inability of the somatic cells to regenerate or maintain their telomeres, poses a major problem given the lengthy selection process needed to identify a targeting event. The second problem is the overall inefficiency of homologous recombination. A double strand break or introduction of foreign DNA into a cell can be processed either through the homologous recombination or non-homologous end joining pathways. Of these two, non-homologous end joining is dominant in somatic cells. A two plasmid recombination system was used to study the effects of the manipulation of several non-homologous end joining and homologous recombination pathway molecules on the rates of homologous recombination in porcine fetal fibroblasts. In addition, the effect of telomerase expression, cell synchrony, and

DNA nuclear delivery was examined. Results indicate a strong positive relationship between inactivation of p53, cell synchronization, and efficient DNA nuclear delivery in enhancing the rate of homologous recombination. These findings were then applied to an actual locus in the pig, the $\alpha 1,3$ galactosyltransferase gene. Results from these transfections are compared to published accounts of successful targeting at this locus and possibilities for the differences found are discussed.

DEDICATION

To my amazing parents, Steve and Rose Marie Zaunbrecher.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

BACKGROUND

Project Overview

While the efficiency of targeting genes in mouse ES cells averages approximately 1×10^{-6} , in somatic cells (fetal fibroblasts) targeting efficiency has been estimated to be 10 to 100 times less (1-5). This low efficiency is further exacerbated by the high occurrence of random insertions of the transgene. In most current targeting protocols, random insertion events account for the vast majority of colonies arising after selection. This low efficiency combined with the finite lifespan of fibroblasts makes the lengthy process of targeting and identifying positive cells very difficult. Currently, most targeting protocols use traditional types of enhancements to increase targeting efficiencies. These enhancements include promoter/polyA trapping and positive/negative selection. While there are many reports of enhancement achieved using these enrichment schemes, the most realistic values of enhancement of homologous recombination (HR) are in the range of approximately 2-5 fold with positive/negative selection and approximately 100-500 fold with promoter traps (4,6,7).

When a cell experiences a DNA double strand break (DSB), two competing

This dissertation follows the style of *Nucleic Acids Research*.

pathways are activated, the HR pathway and the non-homologous end joining (NHEJ) pathway. In cells such as yeast and ES cells, the HR pathway appears to be dominant, while in somatic cells the NHEJ pathway appears to dominate the correction of the DSB (8).

While there are many proteins involved in NHEJ, the most studied have been Ku70, Ku80, DNA dependent protein kinase catalytic subunit (DNA-PKcs), and the Rad50/Mre11/Xrs2 protein complex. A DSB occurring at the beginning of S phase will be first recognized and the free DNA ends bound by the Ku70/Ku80 heterodimer. This binding recruits DNA-PKcs which attracts a cascade of proteins to the sight of the break. The protein complex of Rad50/Mre11/Xrs2, which possess helicase and endonuclease activity, will bind to the free DNA ends and make them more accessible for the proteins involved in aligning the areas of micro-homology needed for DNA ligase IV to bind the ends together (9).

When a DSB is repaired through HR, Rad51 protein binds to the free 3' ends of the DNA at the break site made by the actions of the Rad50/Mre11/Xrs2 protein complex mentioned above. With the help of the many proteins in the Rad52 epistasis group, Rad51 interacts with undamaged DNA to search for a large area of homology. Once an area of homology is found, Rad51, and associated proteins, initiate strand exchange, strand invasion, and strand resolution resulting in the repair of the DSB (9) (Fig. 1). Many studies have examined over-expression of several HR proteins and their effects on HR rates. An example of this would be the studies by Vispe et al. (10) who

found that a two-three-fold over expression of Rad51 in Chinese Hamster Ovary (CHO) fibroblasts resulted in a 20-fold increase in the rates of HR. However, there are no reports of studies on suppression of NHEJ pathway molecules and its effects on the rates of HR. We examined different molecules including a truncated Mre11 protein. The truncated protein contains the binding site for Ku70 protein (11) and can sequester Ku70 thus inhibiting its binding to Ku80 to form a functional heterodimer. We hypothesized that this in turn would cause down regulation of the NHEJ pathway. Similarly, an RNA aptamer that contains a Ku70/80 specific binding sequence was also tested. The anti-Ku aptamer will bind to the Ku70/80 protein complex and inhibit its binding to DNA which in turn should down regulate DNA-PKcs phosphorylation (12). The final transiently expressed molecule was a mutated p53 protein. This p53 mutation is identical to the mutation found in several cancers and is hypothesized to disrupt the p53 tetramer's ability to arrest the cell cycle at late G1/early S phase therefore bypassing more quickly the time in the cell cycle when the NHEJ pathway is prevalent. The HR pathway, more prevalent in late S phase, would then be able to repair the DSB that would normally be repaired by NHEJ. Previous studies (13-16) have shown that wild type p53 interacts with numerous molecules involved the HR pathway including Rad51, Rad54, BLM and WRN (the proteins responsible for Bloom and Werner syndrome, respectively), as well as stalled replication forks. In addition, there have been several reports of inactivated p53's ability to enhance HR in a variety of mammalian cells (17-21). The mutation proposed corresponds to the proposed binding site found for p53 and Rad51 (13). Theoretically, the transiently expressed dominant negative mutant p53 molecule should

allow continuation of the cell cycle and an increase in the availability of Rad51 which, in turn, should result in an increase in the rates of HR.

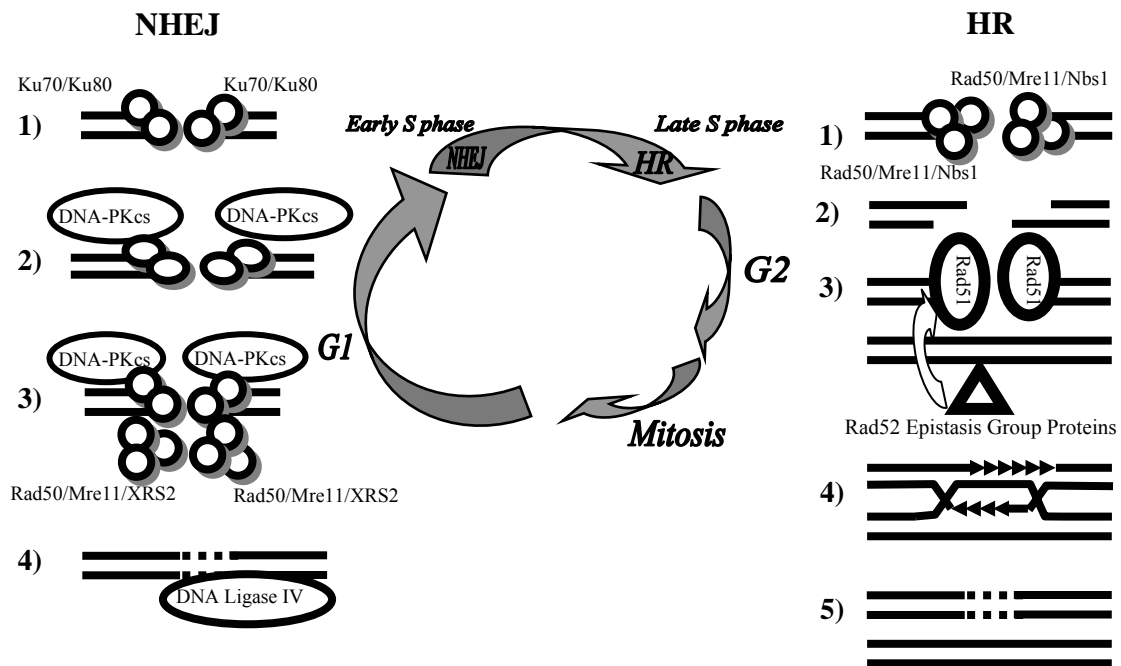


Figure 1. Schematic of the two pathways to repair a DSB occurring during the S phase of the cell cycle.

In addition to testing modifiers of the NHEJ and HR pathways we tested the effect of telomerase expression, cell synchrony, and nuclear delivery of DNA on HR. While there seems to be an association between telomerase expression and the rate of HR (22-24) there has not been a well-controlled study designed to quantify this relationship. Other parameters that may influence the rate of HR are cell synchrony and the timing of DNA entry into the nucleus. Unlike NHEJ, which occurs early in the S phase of the cell cycle, HR occurs late in the S phase. Yet commonly used targeting

vectors are, to a large extent, excluded from the nucleus until nuclear envelope breakdown occurs during the M phase of the cell cycle. After these protocols are tested, the most promising ones are used to target an actual locus, the $\alpha 1,3$ galactosyltransferase gene.

Pronuclear Microinjection

The first methods for introducing foreign DNA into mammalian cells to produce a transgenic animal was by pronuclear microinjection (25,26). This technique relies on DNA delivery to the male and/or female pronuclei of the newly fertilized oocyte. Small amounts (100-1000 copies) of DNA constructs are injected into the pronuclei and the resulting offspring is born (Fig. 2). One major problem with this technique is the development of mosaics. Mosaics occur when the transgene does not enter the genome until after several rounds of cell divisions have occurred, therefore, the resulting offspring does not contain the transgene in every cell of its body. Analysis can show if the transgene was incorporated into the host's genome, how many copies, and in what order (head to tail concatamers). The identified transgenic animals, called founders, are used to produce animals that are non-mosaic transgenics. These F1 offspring are used for further experiments including testing to determine if the transgene is active in the animal and whether it elicits the expected response. While pronuclear injection has served the genetic world, and especially the mouse genetic world, there is a major problem in this technology in that one is severely limited in the range of genetic modifications available. A few of the useful manipulations available to pronuclear

injection include: gain of function, over-expression of a transgene that will either up regulate a physiological process or, down regulation of proteins through a dominant negative mechanism, ribosymes, or anti-sense RNA (27-29). Finally, there is the ability to perform cell ablation experiments to determine cell lineages. (30)

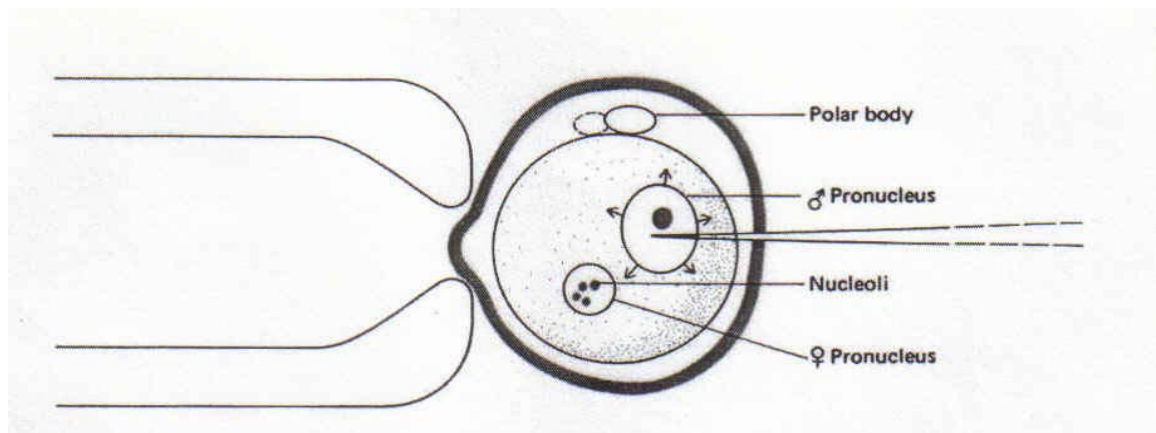


Figure 2. Schematic of microinjection into the male pronucleus of the oocyte [adapted from Hogan et al. (31)]

ES Cells, ES-Like Cells, Targeting, and Chimeras

In contrast to pronuclear injection, more detailed and precise work could be achieved with targeted mutations of particular genes. With the development of embryonic stem (ES) cell technology targeting techniques could be used. In 1981, two groups derived pluripotent cells from the inner cell mass (ICM) of mouse blastocysts (32,33). These ES cells were unique in that they could, with proper supplementation, grow in culture indefinitely and maintain both their normal karyotype and undifferentiated state. However, given the proper stimulus, ES cells could be driven *in vitro* to develop into various cell types (for review see 34,35) ES cells were first used

for early development and cell lineage studies until 1984 when Bradley et al. (36) demonstrated the ability of ES cells to colonize the germ line of chimeric mice produced from the injection of the ES cells into donor blastocysts. In 1987, Thomas and Capecchi (37) were the first to demonstrate the ability to introduce a targeted mutation in ES cells through homologous recombination (HR) of the hypoxanthine phosphoribosyl transferase (HPRT) gene. Transgenic ES cells, usually derived from the inbred mouse strain 129 or its substrains, are injected into developing blastocysts, usually from the BALB/c strains. To aid in the identification of chimeras the two strains have different coat colors. The resulting offspring (F_0) should have both strains' cell types in all tissues including the germline. To test for germline transmission the F_0 mice are bred to the same strain as the blastocyst donors. The resulting offspring (F_1) will either be purebred blastocyst donor strain or will be a full hybrid of the ES cell and blastocyst donor strains. If the transgene is not detrimental to the animal and follows normal Mendelian genetics, one half of the hybrids will not contain the transgene while the other half will be heterozygous for it. Diagnostic tests such as Southern blot analysis are then performed to determine which hybrid F_1 contains the transgene. Once these animals are identified, crossing strategies will ultimately result in an animal homozygous for the transgene. Alternatively, due to the proliferative nature of ES cells, F_1 offspring ES cells can be used for a second round of targeting using a second selectable marker and the appropriate precautions followed by another round of blastocyst injection, F_0 , and F_1 offspring to yield a homozygous transgenic mouse (Fig. 3).

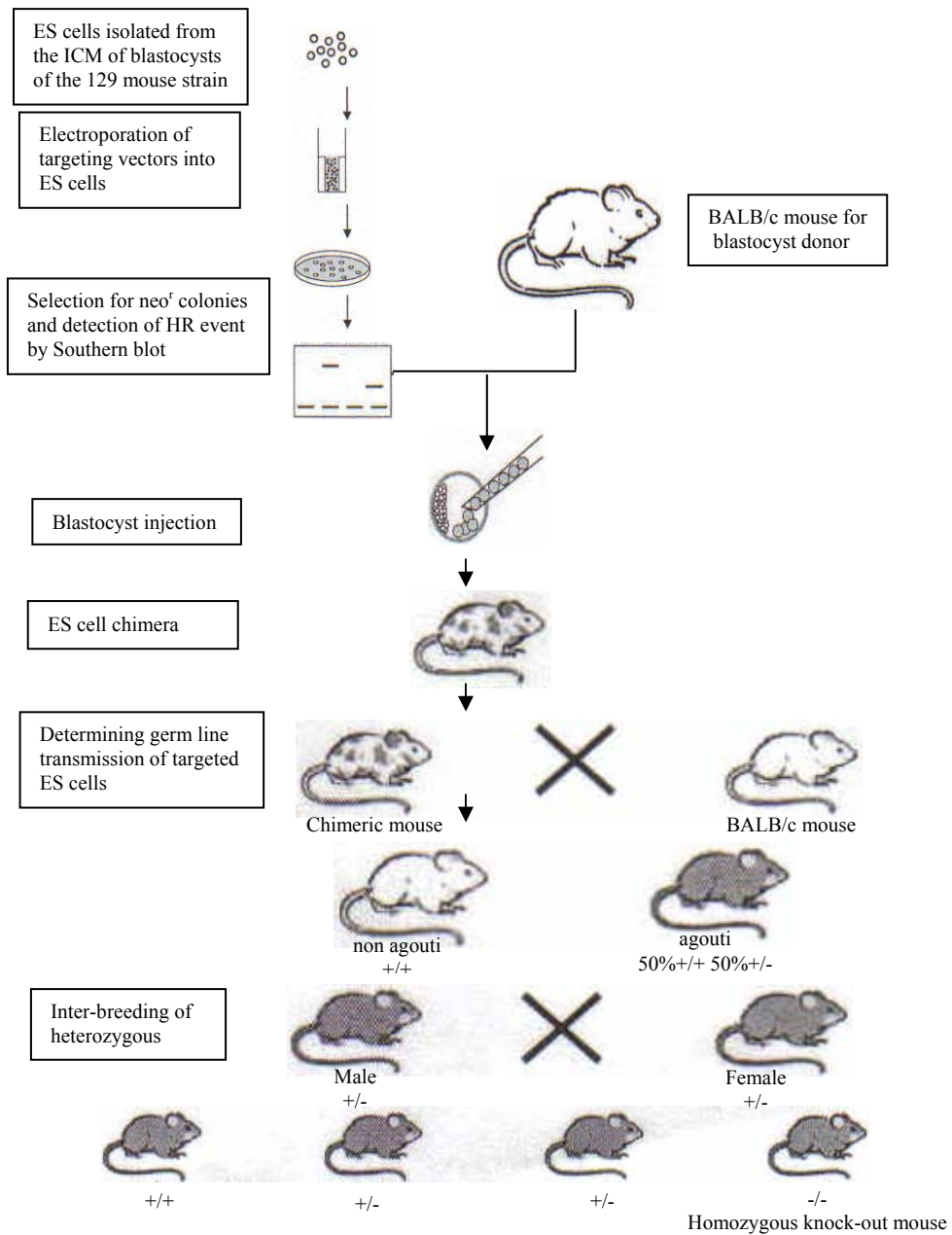


Figure 3. Generation of transgenic mice from targeted ES cells. [Adapted from Prella et al. (38)]

Another class of pluripotent cells was soon developed from the urogenital ridge of developing fetuses. These primordial germ cells (PGCs) or embryonic germ (EG) cells share many of the characteristics of ES cells including high nuclear: cytoplasm ratio, growth in compact multilayer colonies, alkaline phosphatase activity, highly conserved epitope markers such as stage specific embryonic antigen-1 (SSEA-1) and germ line-specific transcription factor Oct-4, high levels of telomerase, ability to maintain stable karyotypes and remain undifferentiated in culture, and the ability to colonize the germ line in chimeras (39-44). With this success of ES, EG, and ES-like cells in mice, an intense effort was directed toward developing pluripotent embryonic cell lines from livestock species.

In cattle, undifferentiated cells have been isolated from all stages of embryonic development from 16-cell to hatched blastocyst. Yet, despite exhaustive attempts there has only been limited success in long term proliferation of undifferentiated bovine ES cells (44-47). One group was able to grow several ES-like cell lines for more than 50 passages and still maintain pluripotency. Pregnancies were established by nuclear transfer using these cells at a lower passage number. However, all pregnancies were lost before day 60 of pregnancy due to incomplete placental development (48). In addition, various attempts at making germ line chimeras from ES or ES-like cell lines have failed (47). Pluripotent bovine PGCs have been isolated and grown for short periods of time successfully (44,49-51). One healthy calf was born after the re-cloning of nuclear transfer embryos made from PGC karyoplasts. But while early attempts at nuclear

transfer using freshly isolated PGCs were unsuccessful, later attempts have resulted in viable offspring (52,53).

In sheep, all attempts at isolating and growing undifferentiated ES or ES-like cells for more than a few passages have failed (for review see 38,54). While attempts to produce germline chimeras have failed, nuclear transfer of differentiated ICM outgrowths have resulted in live lambs (55-57).

The livestock species studied the most has been the pig. Undifferentiated ICM-derived cell isolation, while difficult in this species, has been achieved by few (58-62). Most of these cell lines have been grown for extended periods of time and maintain most mouse ES cell characteristics. However, there has been no reported chimeric germ line transmission using porcine ES cell lines (63). Undifferentiated porcine PGCs have been isolated and grown, in the correctly supplemented media, for prolonged periods of time (64,65). Some of these were transfected with transgenes and injected into blastocyst to yield live chimeric piglets. While the different transgenes were detected in several tissues, none were found in the germline (64,66).

Cloning Somatic Cells

Because of the difficulties in producing viable ES or ES-like cell lines the advancements of transgenics in livestock has been significantly hindered. However, with the production of Dolly, a viable lamb resulting from the transfer of a nucleus from a somatic cell to an enucleated oocyte, the use of somatic cells as carrier cell lines to produce a transgenic animal could be realized (67). Cloning involves the addition of

somatic cell nuclei (i.e. fibroblast) into the perivitelline space of an enucleated oocyte. An electrical pulse is used to cause the fusion of the cell to the oocyte. Following fusion, the oocyte is activated by electrical or chemical means and the oocyte begins development. The embryo is transferred into a recipient at the correct time of embryo development (i.e. with or without prior culture) and cycle of recipient (variable between species) (Fig. 4) (68,69).

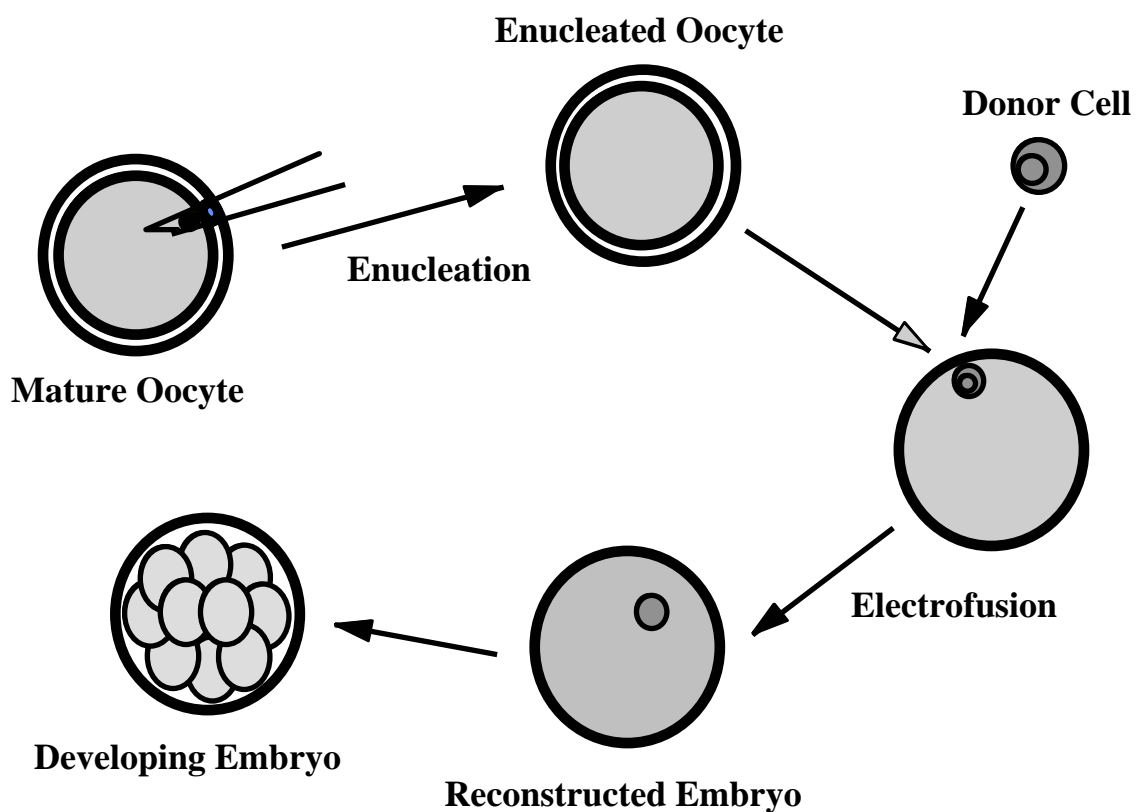


Figure 4. Schematic of nuclear transfer. After enucleation of the mature oocyte and fusion with a donor cell nucleus, the reconstituted embryo is capable of normal development.

There are many advantages of cloning over that of pronuclear injection and chimeras. The most obvious is that every cell of the resulting transgenic animal contains the transgene, this dramatically reduces both the analysis and time involved in producing a completely transgenic animal. However, there are also many pitfalls in this technology. The efficiency of cloning livestock species is very low due to high embryo loss, developmental problems, and to a lesser extent, post-parturition losses (67,70). This is further exaggerated with the inefficiencies of gene targeting in somatic cells. The main reasons for this inefficiency are the finite life span of the fibroblasts used in cloning and the inefficiency of these cells to undergo specific genetic changes. However, cloning of targeted livestock fibroblast was successfully accomplished with the birth of sheep, targeted at the COL1A1 locus. This demonstrated that it was indeed possible to make the considerable manipulations needed for targeting, and still have the capability of producing viable offspring (70). Most recently, three groups (71-73) have reported successful targeting of the $\alpha 1,3$ galactosyltransferase (GT), this gene encodes for an enzyme which is responsible for adding a carbohydrate onto the epithelial cells of blood vessels that cause hyperacute rejection when introduced to antibodies present in human blood (74). In addition, Phelps et al. (75) have reported the generation of the first homozygous $\alpha 1,3$ galactosyltransferase null pig through a spontaneous mutation of their GT+/- cell line. Yet, HR in livestock somatic cells remains a formidable task and to date all targeted genes have used promoter trap enrichment protocols requiring expression of the target gene. Methods for enhancing homologous recombination in somatic cells, therefore, would facilitate the development of transgenic livestock.

Experimental Molecules

Mre11/Rad50/Xrs2(Nbs1) Protein Complex

Mre11, Ku70/80, and p53 were selected for manipulation and analysis. Mre11 is an 80kDa multifunctional protein that forms the central binding core of the Rad50/Mre11/Xrs2 protein complex. Mre11 possesses 3' to 5' double stranded (ds)DNA exonuclease and both single stranded (ss)DNA and dsDNA endonuclease activities. The different nuclease activities are structure specific in that the dsDNA exonuclease activity is only active in substrates containing blunt or 5' overhangs, and is much less active with 3' overhangs (76-80). In addition, the binding of Mre11 to various repair molecules affects the nuclease ability of Mre11 in a species specific manner (81). Studies by de Jager et al. (82) and Chen et al. (83) have shown that the Mre11 complex preferentially binds to the ends of linear DNA molecules. The second member of the Mre11 complex is the Rad50 protein. Rad50 is an approximately 150kDa ATP-dependent protein whose likely function is to help bring the DNA ends together and hold them in loose proximity for their subsequent repair. This function helps to facilitate the search for short and long regions of sequence homology in the recombining DNA templates, which is crucial for DSB repair in both NHEJ and HR. This close proximity of the DNA ends also helps to stimulate DNA ligase activity. Additionally, the association acts as a bridge connecting the broken chromatid and its sister so that there won't be separation between the two. Finally, the position of Rad50 on the DNA ends helps to protect the DNA ends from the nuclease activities of Mre11 (81-84). The final protein in the Mre11 complex is the Xrs2 protein (yeast) or the Nbs1 protein (vertebrate

homologue). Because of the divergent homologies between the yeast and vertebrate proteins, a defined function has been elusive. However, there is a Mre11 binding site on the extreme carboxy terminus of Nbs1 and a Nbs1 binding site on the amino terminal half of the Mre11 protein (85).

Studies examining the distribution of the Mre11 complex within the cell show homogeneous distribution within the nucleus in resting and undamaged cells, but association in sites of DNA damage within 30 minutes of treatment with genotoxins to form nuclear foci of Mre11 complexes (86,87). Interestingly, only agents that form DSBs show Mre11 foci while other DNA damaging agents such as UV light do not (87,88). Evidence has accumulated suggesting a role of the Mre11 complex in replication fork DSB repair mechanisms. Studies by Costanzo et al. (89) have shown that cells with non-functional Mre11 complexes exhibit higher rates of DSBs during replication. These results shed light on the reasons why Mre11 foci are seen in undamaged S phase cells. The most probable role of Mre11 complexes during replication is to prevent DSBs by resolving hairpin loops in DNA that tend to stall replication forks and thus form DSBs (81).

The role of the Rad50/Mre11/Nbs1 complex in repairing DSBs is controversial. In *Saccharomyces cerevisiae* Rad50 and Mre11 proteins are essential for DSB end joining in the NHEJ pathway (90,91) but in *Schizosaccharomyces pombe* they have no role in DSB repair (92). In higher eukaryotes it is difficult to access the role of the complex because deletions of either Mre11 or Nbs1 lead to loss of functions essential for normal cell growth (93-95). Natural human mutations of Nbs1 and Mre11, in Nijmegen

breakage syndrome and a variant of ataxia telangiectasia respectively, have been shown to be sensitive to radiation, but do not appear to have defects in DSB repair itself (96-98). Huang and Dynan (99), using cell free end-joining systems, have shown a requirement for the Rad50/Mre11/Nbs1 complex in mammalian DNA NHEJ pathway. Taken together, the Mre11 complex is most likely involved in DSB repair in both NHEJ and HR but possibly plays a minor role in helping to keep the two DNA ends together for the repair machinery to function properly.

Ku70/80 Protein Complex

The second molecule investigated is the Ku70/80 heterodimer protein complex. Much of what is known about the NHEJ pathway molecules have been from the study of yeast and mammalian cells lacking in various NHEJ and HR pathway molecules. In 2001, Walker et al. (100) reported the crystal structure of the Ku heterodimer and helped to clarify the suspected roles of the Ku70/80 heterodimer. Ku has been shown to bind tightly and specifically to a variety of DNA ends including blunt ends, 5' and 3' overhangs, as well as hairpins (101,102) suggesting that Ku70/80 is the molecule that initially binds to the DNA ends of a DSB and recruits DNA-PKcs. In binding, Ku protects the DNA ends from excessive nuclease degradation and also helps to align the two broken ends of DNA. Ku may also play a role in targeting DNA DSBs to appropriate sites for repair (103). Finally, there is strong evidence that Ku protein plays an important role in telomere maintenance (104).

p53 Protein

The final molecule investigated is the p53 molecule. p53 is a complex, multi-functional protein. One important physiological function is to curb cell cycle progression or induce apoptosis following certain types of DNA damage. Results of various experiments with inactive p53 suggest that p53 protects the cell from genomic instability. In the event of DNA damage from harmful agents, introduction of nucleases into the nuclei, or even DNA transfection, p53 accumulates and blocks entry into the S phase of the cell cycle. This delay at G1 will continue until repair of the damaged DNA has occurred or, if the damage is too extensive, p53 will trigger apoptosis and destroy the cell (105-107). However, when cells are defective in p53 there is no arrest at G1. Studies by Livingstone et al. (108) have shown that a large fraction of p53 negative mouse embryo fibroblasts showed aneuploidy at passage three in culture. In addition, Harvey et al. (109) found a higher incidence of cells with abnormal chromosome numbers in mouse embryonic fibroblasts heterozygous for an inactive p53 allele but not until passage twenty five. However, more recent work by Bunz et al. (110) have shown that the precise inactivation of the p53 gene by HR does not result in aneuploidy in human colon cancer cells and human fetal lung fibroblasts. However, they did detect a slight trend to tetraploidy. They concluded that p53 inactivation alone is not the cause of aneuploidy, but the absence of p53 can exacerbate pre-existing tendencies toward chromosome instability.

Targeting Plasmid Design

The ability to precisely modify a gene through targeting is a powerful tool and enables the researcher to study a multitude of phenomena (111,112). However, despite the many applications of targeting, there are basically two types of targeting vectors used in gene targeting, the insertion vector or O-type and the replacement vector or Ω -type. The O-type vector is linearized within an area of homology to the target gene. Incorporation will result in the duplication in the endogenous gene of the homologous DNA present in the vector as well as the insertion of the plasmid backbone. The Ω -type vector is linearized outside the area of homology and incorporation results in only the replacement of endogenous sequences with the “same” exogenous sequence from the targeting vector, usually flanking a selectable marker (Fig. 5). While insertion type vectors tend to yield higher recombination rates, the possible problems with the addition of the exogenous plasmid DNA can be avoided using the replacement vector. Therefore, most laboratories use the replacement vectors, with the addition of enrichment schemes, in their targeting protocols (113).

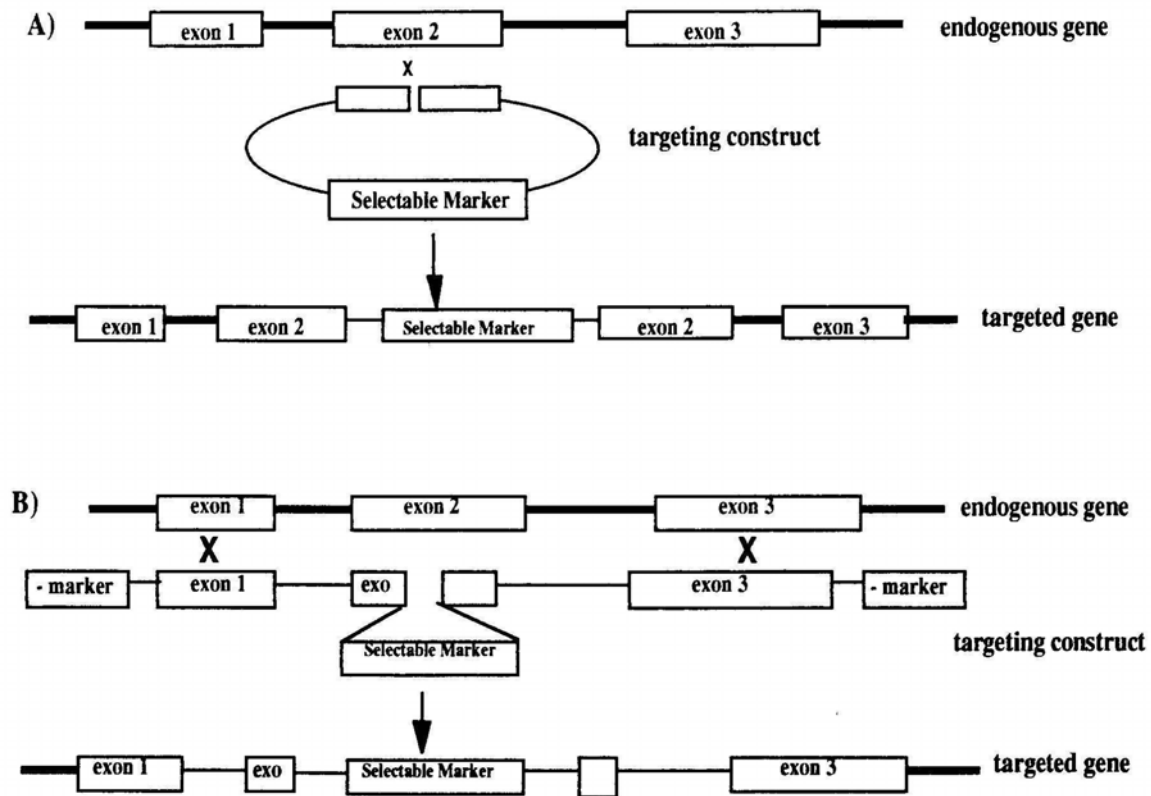


Figure 5. Schematic of the two types of homologous recombination. A) Insertion or O-type homologous recombination B) Replacement or Ω-type homologous recombination. (exo=fragment of exon 2)

Promoter Trap Enrichment

The enrichment schemes most used today are the promoter trap, polyA trap, and positive/negative selection (PNS). The most popular enrichment scheme is the use of a promoter trap in the targeting construct. The promoter region of the selectable marker of a replacement vectors is removed and the marker is inserted inside an exon, in frame with the coding region. When incorporation occurs and the targeted gene is transcribed and translated, a fusion protein of the target gene and selectable marker results. The

transformed cells can then be selected with the appropriate selection media and the targeted cells recovered. However, there are some problems with this technique. While promoter trapping enriches the targeting system by approximately 100 fold (6) there is still the possibility of the target construct randomly inserting in frame into another active gene therefore giving a false positive. In addition, the fused protein may inhibit the selectable marker's ability to function correctly. Finally, only actively expressing genes are available for targeting with this method. To help prevent problems with fused protein interference with the selectable marker expression, most protocols involve the addition of an internal ribosomal entry site upstream of the selectable marker.

Internal Ribosomal Entry Site

During normal eukaryotic translation, various initiation factors assemble initiator tRNA, 40S, and 60S ribosomal subunits into a functional 80S ribosome at the initiation codon of mRNA. In picornaviruses such as encephalomyocarditis virus (EMCV), Theiler's murine encephalomyelitis virus (TMEV), and foot-and-mouth disease virus (FMDV) an internal ribosomal entry site (IRES) is responsible for the initiation of mRNA translation (114). The EMCV, most commonly used in targeting vectors, is an approximately 400 nucleotide sequence 5' of the initiation codon. The RNA forms a distinct secondary structure that consists of four major structural domains. No significant sequence or structural similarities have been found between IRES's of unrelated viruses and studies have shown various and unique requirements for each class of IRES (114).

In addition, Macejak and Sarnow in 1991 (115) discovered the first cellular IRES in the 220 nucleotide long 5' untranslated region of the immunoglobulin heavy chain binding protein mRNA. Since then, many IRES-like acting sequences have been found in eukaryotic cells as diverse as yeast to mammalian cells (116). The most probable reason for the presence of an alternative method of translation for specific genes is likely as a safety mechanism to ensure the cell's ability to produce proteins during times of impaired cap-dependent translation. Such times include physiological impairment of the cap mediated translation during mitosis, quiescence, differentiation, and stress or when proteolytic enzymes cleave necessary initiation factors during apoptosis or viral infection (116,117).

The use of the EMCV IRES element was first used by Ghattas et al. in 1991 (118). The dicistronic expression vector was introduced into chick embryos and successfully translated both β -galactosidase and the chloramphenicol acetyltransferase gene (CAT). Since then it has been used extensively in the field of gene trapping and has helped in the discovery of countless new genes (119). In addition, the presence of an IRES in a promoterless expression vector has been shown to increase the efficiency of trapping novel genes by as much as 15 fold (119). This enhancement is mainly due to two reasons. First, because the IRES allows for cap-independent translation, the downstream protein is not fused with the protein from which the promoter drives. Second, when using a promoterless construct, normally the exogenous gene must be in frame with its fused protein. This is not a requirement due to the presence of the IRES. Therefore, the properly designed plasmid must only integrate into an actively transcribed

gene for correct and detectable expression of the gene of interest to occur, even in very low expressing genes (119).

With the success of IRES in gene trapping Wood et al. (120) incorporated the same concept into targeting vectors. Placing an IRES 5' of the promoterless selectable marker allowed for similar enhancement in targeting genes in ES cells as it did in trapping genes. Research has shown that IRES containing constructs are active in a variety of loci that exhibit various levels of expression and have been shown capable of being transmitted successfully through the germline (121,122).

PolyA Trap Enrichment

The polyA trap enrichment protocol can be useful in targeting non-expressing genes. This method relies on trapping the target gene's polyA signal. The selectable marker is driven by its own promoter but lacks a polyA signal. Upon incorporation into the target sequence, the selectable marker will be expressed. Because of the high background of random targets due to the polyA trap plasmid incorporating in any gene, the enrichment with this protocol is not as high as the others, and is seldom used (4,6,7)

Positive/Negative Selection Enrichment

A positive/negative selection protocol involves the use of either the positive selection protocols mentioned above with the addition of a negative selectable marker outside the area of homology. It has been shown that homologous recombination will result in incorporation of only the homologous exogenous DNA including the flanked

positive selectable marker and will exclude the negative selectable marker. However, random integration of the construct will result in the incorporation of the entire targeting construct including the negative selectable marker. Proper selection techniques should result in cells that survive only if the positive marker is active and the negative selectable marker is inactive or not present. Problems arise with this technique when random incorporation results in the inactivation of the negative selectable marker and therefore the overall enrichment usually received from the use of PNS is about two to five fold (4).

Non-Traditional Enhancements

Telomerase

Telomeres are specialized DNA/protein structures located at the ends of chromosomes. Many proteins have been found to associate with telomeres and are thought to act as protective caps to prevent chromosome fusion and to distinguish the chromosome ends from DSBs in need of repair. Surprisingly, many of the proteins involved in NHEJ such as Ku70/Ku80, DNA-PKcs, DNA Ligase IV/XRCC4, and Rad50/Mre11/Nbs1 have been shown to have intimate contact with telomeres. Studies by d'Adda et al. (123) have shown that Ku70/80 and DNA-PKcs are bound to telomeres in both telomerase positive and ALT cells, cells that maintain telomeres through HR mechanisms. In addition, using KU80^{-/-} or Ku70^{-/-} mouse cells they have shown that both exhibit dramatically shorter telomeres than Ku^{+/+} strains and that Ku^{+/-} strains show an intermediate shortening. Finally, they found that null mutations of either component of the XRCC4/DNA Ligase IV complex showed no shortening of telomeres.

Alternatively, Hande et al. (124) used *scid* mice, lacking in DNA-PKcs, to show that these mice exhibited longer telomeres than their wild type genetic match controls. It has been shown previously that the Ku complex and essentially all of the cellular TRF1, a mammalian telomere binding protein, localized at the telomere (125). Hsu et al. (126) expanded on this and found that the Ku complex does not bind to the telomere sequence directly but to the TRF1 which in turn binds the telomere. Peterson et al. (127) have shown that the Ku70/80 complex interacts with a stem loop structure present on the telomerase RNA gene product. The over-expression of this structure in normal cells resulted in telomere shortening and loss of telomeric silencing, phenotypes similar to those seen when the Ku complex is dysfunctional (128). Over-expression of the stem loop structure in cells lacking either Ku70 or Ku80 had no impact on telomere length and only addition of both complex components resulted in the reverting back to shortened telomeres and lack of telomeric silencing. They could not demonstrate, however, if the stem loop interacted directly with the Ku complex. The model proposed from this work is that the stem loop, which does not appear to directly participate in catalysis telomere addition, interacts with the telomerically bound Ku which helps to keep telomerase in close proximity to the chromosome end. Ku could then pass the telomerase to proteins such as Cdc13p and/or Est1p, which have been implicated in telomerase recruitment and are required for telomere addition *in vivo*. Alternatively, the interaction between the stem loop and Ku could activate telomerase after it has been brought to the telomere.

Evidence has shown that the Ku complex is the primary sensor of DSBs during NHEJ and the binding of the complex to DNA triggers the activation of the protein cascade needed for repair of the DSB (9,102,129,130). Evidence has also shown that cells high in telomerase activity such as ES cells, regenerating liver cells, and Fanconi's anemia cells show higher rates of HR and lower rates of NHEJ than somatic cells not expressing telomerase (1-5,22-24). Could the sequestering of Ku by the telomeres/telomerase cause a shift in the repair of DSB from NHEJ to HR? To date, there have been no reports on the relationship between the ectopic expression of telomerase and the rates of HR in normally telomerase negative somatic cells.

Nuclear Localization Signal

More efficient delivery of the transgene to the nucleus would decrease time in the cytoplasm where it could be degraded. In addition, rapid delivery to the nucleus allows for longer exposure of the transgene to the HR machinery than if cell division and nuclear membrane breakdown is required. Studies of nuclear localization of cytoplasmic proteins to the nucleus have shown that these proteins possess a nuclear localization signal (NLS) that interacts with cytoplasmic proteins which transport the proteins to the nucleus via a nuclear pore complex (NPC) (131). In addition, Vacik et al. (132) has shown that plasmid DNA containing a 366bp sequence from simian virus 40 (SV40) origin of replication and early promoter were bound by cytoplasmic transcription factors that contain an NLS sequence, and were transported into the nucleus through the NPC.

Synchronization

An alternative method to enhance HR would be to have the majority of the cells synchronized at a point in the cell cycle, mid-late S phase, where HR is favored over NHEJ. It is well established in mammalian cells that compounds that inhibit replication are strong inducers of sister chromatid exchange, gene amplification, and HR (133-137). In addition, Saintigny et al. (138) showed repair of DSBs during replication fork stalling during early S phase to be associated with NHEJ and breaks occurring at the latter stage of S phase to be corrected by HR. While studies by Lundin et al. (139) did not show DSB present when cells were arrested with thymidine, they did indicate that HR of the HPRT gene in Chinese hamster cell lines was the sole mechanism responsible for rescuing cells arrested in S phase by thymidine incorporation. However, a comparison of HR rates between normal and thymidine blocked cells has not been reported.

Objectives

Even though there have been several reports of gene targeting in livestock somatic cells, the efficiencies are still low enough to discourage the general use of this technology. Therefore, any improvement in the area of targeting would help to make it more accessible to investigators. The overall goal of the following experiments was to develop a highly efficient protocol for gene targeting in somatic cells. Using a two plasmid recombination system, the effects of the addition of a truncated Mre11 protein, an anti-Ku RNA aptamer, or a mutated p53 protein on HR in porcine fetal fibroblasts was assayed. In addition, the effects of ectopic expression of human telomerase, and the

interaction as well as separate effects of cell synchronicity and efficient nuclear delivery of DNA will also be assayed. Finally, using the best protocol, a preliminary experiment using an α -1,3-galactosyltransferase targeting vector was performed to determine if the protocol works on an actual locus in porcine fetal fibroblasts.

CHAPTER II

**EFFECTS OF THE MANIPULATION OF NON-HOMOLOGOUS END JOINING
AND HOMOLOGOUS RECOMBINATION MOLECULES ON THE RATES OF
HR IN PORCINE FETAL FIBROBLASTS USING A TWO PLASMID
RECOMBINATION SYSTEM**

INTRODUCTION

The first inbred mouse embryonic stem (ES) cells were gene targeted in 1987 when Thomas and Cappechi (37) successfully targeted the hypoxanthine phosphoribosyl transferase (HPRT) gene. Following targeting, a transgenic animal was made through blastocyst injection of the transgenic ES cells to form a chimeric mouse whose germ line contained the transgenic cells and was able to pass the transgene to the next generation, thus producing a transgenic animal. Since then, gene targeting in mouse ES cells has become common place. However, attempts to develop a viable, pluripotent ES cell line in livestock species have had little success (for review see 38,140). With the production of Dolly, a viable lamb resulting from the transfer of a nucleus from a somatic cell to an enucleated oocyte, the use of somatic cells as carrier cell lines to produce a transgenic animal could be realized (67). This possibility was further advanced with the production of the first gene targeted transgenic livestock species. The birth of sheep targeted at the COL1A1 locus, demonstrated that it was indeed possible to make the considerable manipulations needed for gene targeting, and still have the capability of producing viable

offspring (70). Most recently, three groups (71-73) have reported successful targeting of the $\alpha 1,3$ galactosyltransferase (GT), an enzyme which is responsible for adding a carbohydrate onto the epithelial cells of blood vessels that cause hyperacute rejection in the presence of human blood (74). In addition, Phelps et al. (75) have reported the generation of the first homozygous $\alpha 1,3$ galactosyltransferase null pig through a spontaneous mutation of their GT $^{+/-}$ cell line. Yet, HR in somatic cells remains a formidable task and to date all targeted genes have used promoter trap enrichment protocols, requiring expression of the target gene.

Many studies have looked at over-expression of several HR proteins and their effects on HR rates. However, there has been no report of studies on suppression of NHEJ pathway molecules and its affects on the rates of HR. Therefore, the effect of several molecules affecting the NHEJ pathway on the rate of HR was assayed via a two plasmid recombination system.

MATERIAL AND METHODS

Isolation of Fibroblast Cells

Purebred Duroc gilts on day 35 of pregnancy were hysterectomized and the fetuses removed. The head and viscera were removed and the remaining tissue was minced with a sterile razor blade. The tissue was added to 10ml of 0.05% trypsin (Gibco) supplemented with 0.9mM potassium chloride, 0.9mM dextrose, 0.7mM sodium bicarbonate, 0.1mM EDTA (all from Sigma), and 20mM sodium chloride (EM Science).

The tissue/trypsin solution was shaken at 37°C for 15 minutes a total of three times. After incubation, the supernatant was collected, pooled, and pelleted. The cell pellet was resuspended in DMEM/F12 media (Gibco) supplemented with 10% fetal bovine serum (FBS) and 5% calf serum (CS) (both from Hyclone), 30mM sodium bicarbonate, 0.5mM pyruvic acid, and 2mM N-acetyl-L-cysteine (all from Sigma). In addition, 100U penicillin, 100ug, and 250ng amphotericin (Gibco) were added to inhibit microbe growth. The cells were placed in the appropriate number of 10cm tissue culture plates (Corning) and placed in a 5% CO₂ incubator (Nuaire) at 39°C, allowed to attach and grow to confluency and passed (1:2 or 3). The cells were trypsinized and frozen in 50% FBS, 40% media, and 10% DMSO (Sigma), placed in -80°C overnight and then in liquid nitrogen for long time storage and future use.

Plasmids

Two truncated puromycin acetyltransferase (puro) vectors were designed. Primers specific to either the pBluescript or puromycin acetyltransferase gene (puro) were used to generate the plasmids using a pBluescript (pBS) (Stratagene) vector containing a phosphoglycerate kinase-1 promoter (pgk), puro, and bovine growth hormone polyA sequences for the PCR template (Table 1).

Table 1. PCR primers utilized for generating the two truncated plasmids

Primer name	Sequence	Priming site
3' Δ puro forward primer	GTAAAACGACGGCCAG	pBluescript
3' Δ puro reverse primer	GCTTGCGGGTCATGCAC	puro gene
5' Δ puro forward primer	CCGAGTACAAGCCCACGG	puro gene
5' Δ puro reverse primer	CAGGAAACAGCTATGAC	pBluescript

(all oligonucleotides (except when mentioned) were purchased from Genosys)

PCR conditions for the 3' Δ puro plasmid were initial denaturing at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 15 seconds, annealing at 62°C for 30 seconds, and elongation at 69°C for 2 minutes followed by a final elongation at 69°C for 7 minutes. The same conditions were used for the 5' Δ puro plasmid with the exception of the annealing temperature changed to 58°C. One ul of Expand High Fidelity polymerase mix (Roche), 20mM of each dNTP (Roche), and 1.5mM magnesium chloride was used. The resulting fragments were then cloned into a pCR2.1 vector (Invitrogen).

The 3' Δ puro is a plasmid containing only the pgk promoter and a 3' end truncated puromycin acetyltransferase gene and lacks the poly A signal (Fig. 6). The 5' Δ puro is a plasmid containing a 5' truncated puromycin gene and the poly A signal, but lacks the pgk promoter (Fig. 6). Both plasmids share approximately 580 base pairs (bp) of homology in the puromycin gene. Neither plasmid is able to elicit puromycin resistance when transfected alone into a cell. However, when both plasmids are

introduced into a cell, HR occurs via their shared homology and a fully functional puromycin resistant gene is formed (Fig. 6). Cells that have undergone a successful recombination event will be able to survive in the presence of puromycin. The rates of recombination, therefore, can be calculated by the number of puromycin resistant colonies that arise after selection.

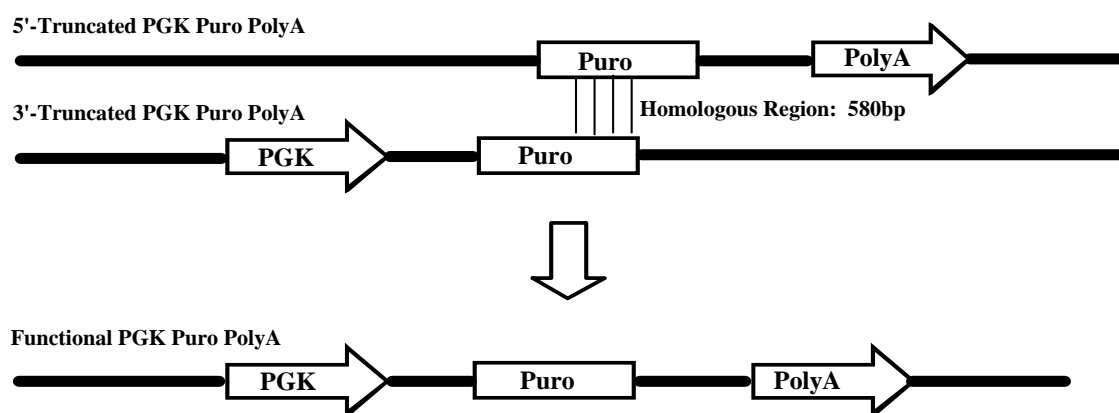


Figure 6. Schematic of the two plasmid recombination system. Neither the 5' nor 3' truncated plasmids alone are able to survive in the presence of puromycin. Both vectors share 580bp of homologous sequence and after HR between the two plasmids, the resulting plasmid is fully functional and will allow for the growth of cell colonies in the presence of puromycin.

The truncated Mre11 plasmid was constructed using porcine fetal fibroblast (PFF) cDNA as the template for primers (Table 2) designed from conserved Mre11 sequences in the mouse (Accession# NM_018736) and human (Accession# BT_006730) which amplified a 408 bp product that encompasses amino acids 24-160 (based on mouse open reading frame [ORF]) using the following PCR conditions: initial denaturing at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 10 seconds, annealing at 55°C for 30 seconds, and elongation at 69°C for 2 minutes followed by a

final elongation at 69°C for 7 minutes. The 408bp PCR product was then cloned into a pCR2.1 vector and re-amplified with the forward primer, Mre11F2, containing homology to the Mre1F1 plus additional NcoI and BspEI sites and the reverse primer, Mre11R2, containing homology to Mre11R1 plus an additional NotI site (Table 2) using the same PCR conditions as before except the annealing temperature was changed to 56°C. The resulting 438 bp product was partially digested with NotI and NcoI (NcoI site 235 bp within amplicon) and subcloned into a pEF/myc/nuc expression plasmid (Invitrogen).

Table 2. PCR primers utilized for generating the truncated Mre11 plasmid

Primer name	Sequence	<u>Additional cut site</u>
Mre11F1	GGATTTATGGAGAAAGATGCAG	none
Mre11R1	CCACAGACATTGAACGTCCAAAG	none
Mre11F2	CGCGCGCCATGGACTCCGGATTTATGGAG	<u>NcoI</u> & <u>BspEI</u>
Mre11R2	CGTATAGCGGCCCGCCACAGACATTGAACG	<u>NotI</u>

An RNA aptamer was purchased from Dharmacon Research, Inc. containing the sequence: A*G*G* UCGGCAUACAGAGUCCGAAUGCGCGUAACUUCG*A*C*U. The oligonucleotide was made in the stable 2'-O-ACE protected form to protect the aptamer from endonucleases and the (*) nucleotides possess a thioate linkage instead of phosphodiester bond for exonuclease protection.

The mutant p53 plasmid was constructed using bovine spleen RNA as a template for reverse transcription using the oligo dT primer. The cDNA was then amplified using hemi-nested primers (Table 3) and the PCR conditions: initial denaturing at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and elongation at 72°C for 2 minutes followed by a final elongation at 72°C for 7 minutes. The resulting fragment was then cloned into a pCR2.1 vector. A Valine¹³⁵ to Alanine¹³⁵ point mutation was introduced following the protocol provided by Clontech and the oligo from Table 3. The mutated cDNA was then subcloned into a pDNA3 under the control of the CMV promoter.

Table 3. PCR primers/oligo utilized for generating the mutated p53 plasmid

Primer name	Sequence
p53 forward primer	ATCCCAGGACGGTGACACC
p53 reverse primer 1	ACCTGCACCAAGCAGAGGTC
p53 reverse primer 2	AGGCTGAGCAGATGAAGACTCC
Mutation oligo	AAGACCTGCCCAG <u>GCG</u> CAGCTGTGGGTC

(GCG is the codon that was changed)

Fibroblasts were re-established from freezing and grown in the (antibiotic free) medium described above. The cells were expanded until appropriate numbers were obtained. At the time of electroporation, cells were trypsinized, counted, and aliquotted to 10×10^6 cells per treatment. Cells were pelleted and resuspended in 0.8ml of cold F10

Nutrient Mixture (HAM) and placed in a 4millimeter gap electroporation cuvette. DNA was delivered into the cells via electroporation (450 volts, 1 millisecond, 4 pulses).

In all experiments, 5nM of the linearized 3' truncated and of the circular 5' truncated plasmid was used. Five nM of the mutated p53 circular plasmid, the Ku RNA aptamer, or the truncated Mre11 circular plasmid was added at the time of electroporation. After each electroporation, the fibroblasts were plated $1-2 \times 10^6$ cells per 10cm tissue culture plate and allowed to recover and attach overnight. Puromycin at the concentration of 2ug/ml was added to all plates approximately 24 hours after electroporation and grown under selection until puromycin resistant colonies were visualized. Plates were washed with phosphate buffered saline and incubated in a 1:1 ratio of methanol:acetone at -20°C for 20 minutes, air dried and stained with trypan blue.

Data Representation and Statistical Analysis

Data sets were transformed, when needed, to aid in acquiring normal distribution of values. Averages of four replications were subjected to ANOVA to determine differences between the means of the different treatments. If a difference was found, Fisher's Least Significant Difference analysis, using SPSS software, was performed to determine which treatments were significantly different at the $p < 0.05$ level. Results are

represented in average colony number of treatment groups (2 plasmids) divided by the average colony number of the positive control (single, fully functional puromycin resistance plasmid) multiplied by 100. This value represents the percentage of colonies that are the result of homologous recombination. The chart shown represents the frequency of HR events per treatment as compared to the control.

RESULTS

Effects of Truncated Mre11, Anti-Ku RNA, and Mutant p53 on the Rates of HR

The results of electroporations consisting of the two truncated plasmids with the addition of one of the repair molecules, truncated Mre11, anti-Ku aptamer, or mutated p53, were compared to the results from electroporations of an intact, functional puromycin resistant gene. The average results of four experiments on the same cell line show no significant differences in HR percentages of the two plasmids between no treatment (control) and the addition of either the truncated Mre11 plasmid or the anti-Ku aptamer. However, a significant difference ($p < 0.05$) was found between the addition of the mutant p53 plasmid and all other treatment groups (Fig. 7).

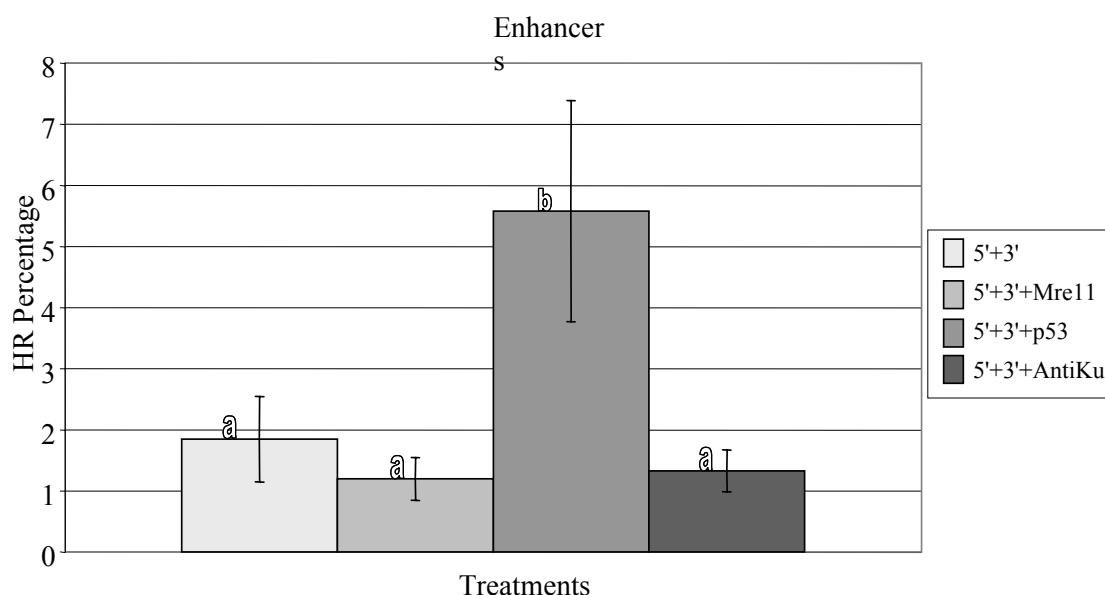


Figure 7. Percentage of colonies resulting from HR of the truncated plasmids with 5nM of the indicated treatment added at the time of electroporation. Error bars = standard error for each treatment group. ($p < 0.05$)

DISCUSSION

Results of this study indicated that manipulation of p53 can result in the enhancement of HR between two truncated plasmids. Similar results were found by Mekeel et al. (18) using HR correction of two mutated hygromycin cassettes in tandem on a single plasmid to calculate HR rates between cell lines that were either positive or negative in p53 activity. The enhancement found in our system is perhaps due to the mutant p53's ability to interfere with both normal p53 tetramer formation and with p53 tetramer/Rad51 interactions. If this were the case, the use of this mutated p53 in targeting protocols would increase the rates of HR by first allowing the cells to bypass

early S phase and also increase the availability of Rad51 in late S phase. Tight control must be exerted on the transient nature of the mutated p53 due to its potential ability to cause aneuploidy in the transfected cells thus making the targeted cells useless for cloning protocols.

Unlike p53, no enhancement of HR was detected with either the truncated Mre11 or the anti-Ku RNA aptamer. Studies by Yoo and Dynan (12) have shown that the RNA aptamer used in our experiments was able to aggressively compete with DNA for the Ku70/80 heterodimer protein complex and, because of this, down regulate DNA-PKcs phosphorylation by 85%. However, Yoo and Dynan (12) used cell extracts and no studies have looked at the relationship between this RNA aptamer and Ku protein *in vivo*. Because no assay for DNA-PKcs phosphorylation was performed in this experiment, it can not be concluded that the RNA aptamer sequestered the Ku70/80 protein in the cells as it did in the cell extracts. Alternatively, if it did work *in vivo* as it did *in vitro*, 15% of the DNA-PKcs in the cell would still be phosphorylated. This meager amount of activity may have been enough to allow for efficient NHEJ to occur. The truncated Mre11 protein studied here represents approximately the first one-fifth of the fully functional Mre11 protein. This portion has been shown to bind to Ku70 protein via a yeast two-hybrid screen (11). In addition, Goedecke et al. (11) found evidence that Mre11 and Ku70 interact naturally in mouse hybridoma cell extracts and co-localize in nuclei of somatic cells and the XY divalent of the mouse testis. However, during early meiotic prophase only Mre11 was abundant and Ku70 was not detectable. They concluded from their data that Ku70 attracts Mre11 to DSBs in order for NHEJ to occur

and in the absence of Ku70 Mre11 is free to participate in HR. There have been no studies to determine if a protein consisting of the first one-fifth of the Mre11 inhibits Ku70's ability to participate in NHEJ. While these results do not determine if it does affect Ku70's involvement in NHEJ, it does show that the truncated Mre11 does not enhance HR between two truncated plasmids.

CHAPTER III

EFFECTS OF TELOMERASE EXPRESSION ON THE RATES OF HOMOLOGOUS RECOMBINATION IN PORCINE FETAL FIBROBLASTS

INTRODUCTION

The ability to further increase HR efficiency in normal somatic cells would have profound effects in the advancements of transgenics. In all successful livestock somatic cell gene targeting reported to date the use of promoter trap enrichment has helped to increase the inherently low levels of HR to that nearly equal to ES cells (70-73,141). While this increase is encouraging it still remains too low to be practically applicable for researchers. There is an interesting phenomenon in cells with high rates of HR such as ES cells, regenerating liver cells, and Fanconi's anemia cells in that they all have been shown to express telomerase (1-5,22-24). Early work by Finn et al. (142) found, using non-functional tandem neomycin sequences, that HR frequencies were higher in immortal cells as compared to normal diploid cells. In addition, Thyagarajan et al. (143) found that cell lysates from immortal cells contained molecules that increased HR between two truncated non-functional neomycin plasmids. More recently, studies have shown an intimate connection between several NHEJ pathway molecules and telomeres and telomerase (123-128) which could play a role of sequestering/down regulation of this pathway and possibly allow the HR pathway to repair DSB in telomerase positive cells. However, to our knowledge, there has not been a well-controlled study designed

to quantify the relationship between the expression of telomerase and the increase in HR efficiency. To this end normal porcine fetal fibroblasts were virally co-transfected with the human telomerase reverse transcriptase (hTERT) cDNA and the green fluorescent protein (GFP). The HR percentage between two non-functional puromycin plasmids in these cells were compared to that of the normal, non-transformed, and GFP-transfected controls.

MATERIAL AND METHODS

Plasmids and hTERT Transformation

Two truncated puro vectors were designed. Primers specific to either the pBluescript or puro gene were used to generate the plasmids using a pBS vector containing a pgk, puro, and bovine growth hormone polyA sequences for the PCR template (Table 1).

PCR conditions for the 3' Δ puro plasmid were initial denaturing at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 15 seconds, annealing at 62°C for 30 seconds, and elongation at 69°C for 2 minutes followed by a final elongation at 69°C for 7 minutes. The same conditions were used for the 5' Δ puro plasmid with the exception of the annealing temperature changed to 58°C. One microliter (ul) of Expand High Fidelity polymerase mix, 20mM of each dNTP, and 1.5mM magnesium chloride was used. The resulting fragments were then cloned into a pCR2.1 vector.

The 3' Δ puro is a plasmid containing only the pgk promoter and a 3' end truncated puromycin acetyltransferase gene and lacks the poly A signal. The 5' Δ puro is a plasmid containing a 5' truncated puromycin gene and the poly A signal but lacks the pgk promoter. Both plasmids share approximately 580bp of homology in the puromycin gene. Neither plasmid is able to elicit puromycin resistance when transfected alone into a cell. However, when both plasmids are introduced into a cell, HR occurs via their shared homology and a fully functional puromycin resistant gene is formed (Fig. 6). Cells that have undergone a successful recombination event will be able to survive in the presence of puromycin. The rates of recombination, therefore, can be calculated by the number of puromycin resistant colonies that arise after selection.

Day 57 PFFs were virally transfected with green fluorescent protein (GFP) alone or with GFP plus human telomerase reverse transcriptase (hTERT) using procedures described elsewhere (144). Functional telomerase activity in the fibroblasts was determined using the TRAPeze Telomerase Detection Kit (Intergen). A population doubling experiment was conducted comparing the GFP and GFP/hTERT expressing cells. Population doublings were calculated using the formula: $[\log_{10}(\# \text{ cells harvested}) - \log_{10}(\# \text{ cells seeded})] \times 3.3219$. In addition, karyotype analysis was performed on both sets of cells. Metaphase spreads were prepared from colchocine arrested cells stained with 4',6-diamidino-phenylindole. Digital images were acquired and analyzed using CYTOVISION software (Applied Imaging Systems).

Fibroblast Cell Culture and Transfections

Pure bred Duroc gilts on day 57 of pregnancy were hysterectomized and the fetuses removed. The skin was removed from the hind leg of the fetuses and the muscle was minced. The tissue was added to 10ml of 0.05% trypsin supplemented with 0.9mM potassium chloride, 0.9mM dextrose, 0.7mM sodium bicarbonate, 0.1mM EDTA, and 20mM sodium chloride. The tissue/trypsin solution was shaken at 37°C for 15 minutes a total of three times. After incubation, the supernatant was collected, pooled, and pelleted. The cell pellet was resuspended in DMEM/F12 media supplemented with 10%FBS and 5%CS, 30mM sodium bicarbonate, 0.5mM pyruvic acid, and 2mM N-acetyl-L-cysteine. In addition, 100U penicillin, 100ug, and 250ng amphotericin were added to inhibit microbe growth. The cells were placed in 10cm tissue culture plates, incubated in a 5% CO₂ incubator at 39°C, allowed to attach and grow to confluency and passed (1:2 or 3). The cells were then trypsinized and frozen in 50% FBS, 40% media, and 10% DMSO for long time storage and future use.

Cells were re-established from freezing and grown in the (antibiotic free) media described above. The cells were expanded until appropriate numbers were obtained. At the time of electroporation, cells were trypsinized, counted, and aliquotted to 10x10⁶ cells per treatment. Cells were pelleted and resuspended in 0.8ml of cold F10 Nutrient Mixture (HAM) and placed in a 4millimeter gap electroporation cuvette. DNA was delivered into the cells via electroporation (450 volts, 1 millisecond, 4 pulses).

In all experiments, 5nM of the linearized 3' truncated and of the circular 5' truncated plasmid was used. After each electroporation, cells were plated 1-2x10⁶

fibroblasts per 10cm tissue culture plate and allowed to recover and attach overnight. Puromycin at the concentration of 2ug/ml was added to all plates approximately 24 hours after electroporation and the cells were grown under selection until puromycin resistant colonies were visualized. Plates were washed with phosphate buffered saline and incubated in a 1:1 ratio of methanol:acetone at -20°C for 20 minutes, air dried, stained with trypan blue, and the colonies counted.

Data Representation and Statistical Analysis

Data sets were transformed, when needed, to aid in acquiring normal distribution of values. Averages of three replications were analyzed by ANOVA to determine differences between the means of the different treatments. If a difference was found, Fisher's Least Significant Difference analysis, using SPSS software, was used to determine which treatments were significantly different at the $p < 0.05$ level. Results are presented as average colony number of treatment groups (2 plasmids) divided by the average colony number of the positive control (single, fully functional puromycin resistance plasmid) multiplied by 100. This value represents the percentage of colonies that are the result of homologous recombination. The chart shown is the frequency of HR events per treatment as compared to the control.

RESULTS

Porcine Fetal Fibroblasts Express Functional hTERT

An hTERT expression plasmid was virally transfected into PFFs and was shown to stably express an active telomerase protein via the TRAPeze Telomerase Detection Kit. Only cell extracts from hTERT transfected cells exhibited the expected laddering pattern after PCR while all other samples, GFP alone transfectants and heat inactivated samples, showed only internal control bands (Fig. 8).

Expression of hTERT Does Not Extend the Lifespan of Porcine Fetal Fibroblasts

A population doubling experiment was also undertaken to determine if this active human telomerase could extend the natural life span of the fibroblasts. To enable selection, a GFP expression vector was co-transfected with the hTERT vector. As a control, GFP only transfected cells were grown and compared to the GFP/hTERT cells. A summary of growth is presented in Figure 9. The human telomerase expressing cells became senescent after approximately 21 doublings while the GFP expressing cells continued dividing to at least 39 population doublings at the end of the experiment. The average number of days between each population doubling was 6 days for the hTERT cells and 4 days for the GFP cells. However, the GFP cells continued to grow robustly while the hTERT cells showed dramatically slower growth at the end of the experiment. As seen in Figure 9, the number of days between each population doubling was still

close to the average for the GFP cells at 3 days while the hTERT cells were close to three times longer than their average days between population doublings at 16 days.

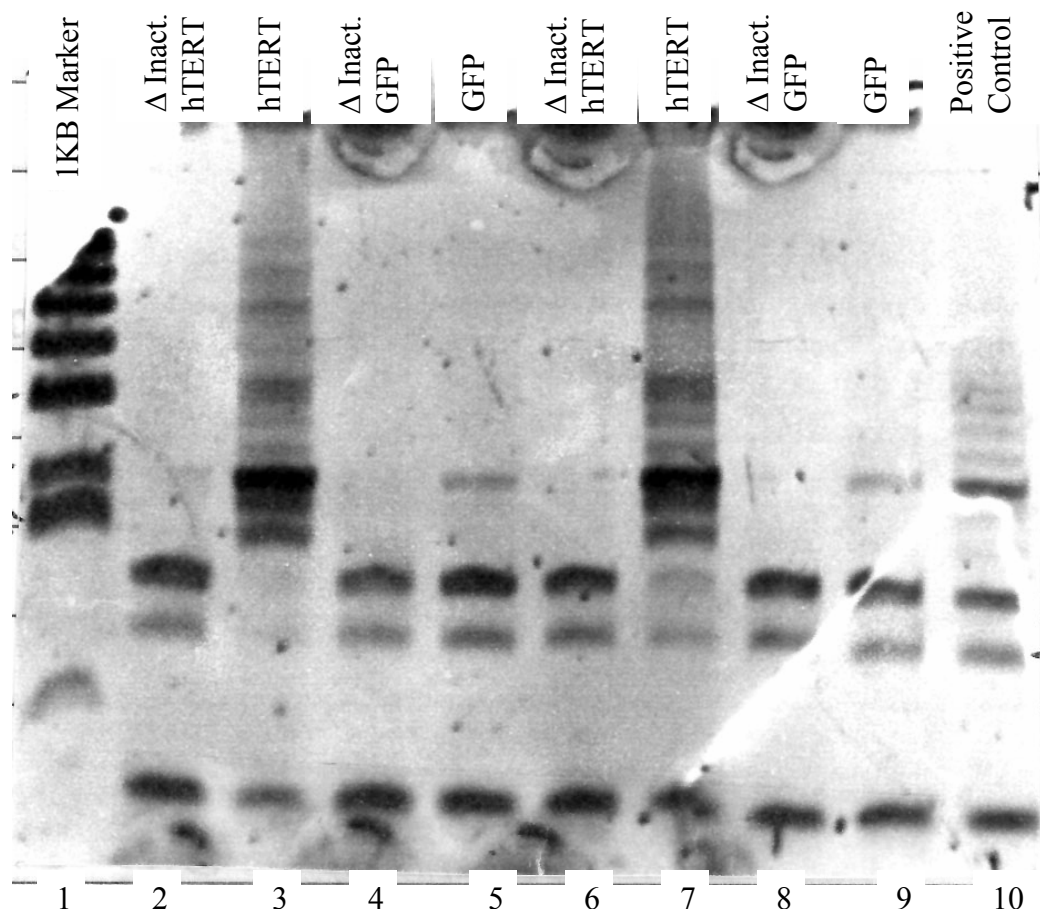


Figure 8. Polyacrylamide gel of TRAPeZ results. Lane 1: 1 kilobase molecular weight marker. Lanes 2, 4, 6, 8: Heat inactivated samples. Lanes 3 and 7: hTERT transfected cell extracts. Lanes 5 and 9: GFP alone transfected cell extracts. Lane 10: Positive control supplied by kit.

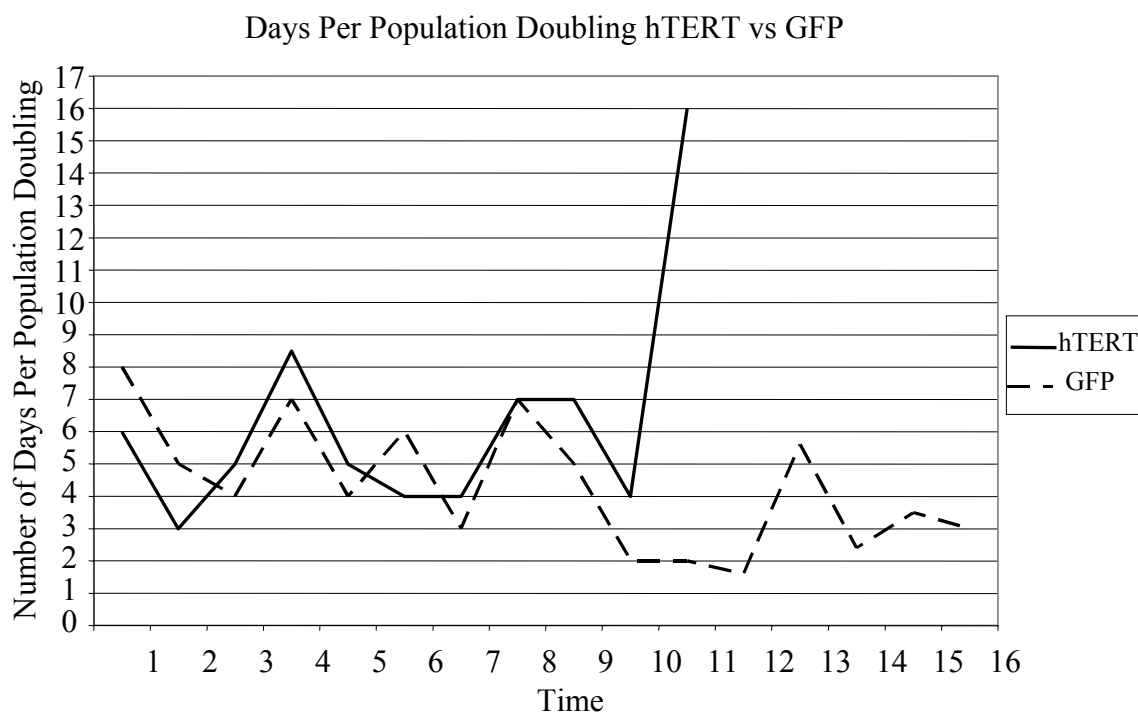


Figure 9. Comparison of the days between population doublings between hTERT/GFP and GFP only expressing cells. GFP only expressing cells continued to have close to average (4) days between population doublings at the end of the experiment while the hTERT/GFP expressing cells had close to three times longer interval between population doublings as the average (6).

Expression of hTERT Does Not Cause Aneuploidy in Porcine Fetal Fibroblasts

Metaphase spreads of the hTERT expressing cells showed 25% of the cells with normal diploid cells ($2n=38$), 25% of cells with variable number of chromosomes (not tri- or tetraploid), and 50% tetraploid cells. While all the GFP cells contained small extra fragments that did not resemble normal chromosomes, 15% were determined to be diploid, 50% were triploid with extra small fragments, and 35% aneuploids of variable number with all being over 57 chromosomes. Figure 10 shows a representative slide of a normal karyotype from the hTERT expressing cell line.



Figure 10. Metaphase spread of an hTERT cell showing normal karyotype $2n=38$.

Expression of hTERT Does Not Increase HR Efficiency in Porcine Fetal Fibroblasts

In order to determine if hTERT expression effected HR, the two plasmid recombination rates between hTERT/GFP expressing, GFP alone expressing, and normal non-transfected cells were compared. Average results of three experiments on the same cell line show no significant differences ($p>0.05$) in the rates of HR of the two plasmids comparing wild type cells and the same cells expressing GFP or GFP and hTERT (Fig. 11).

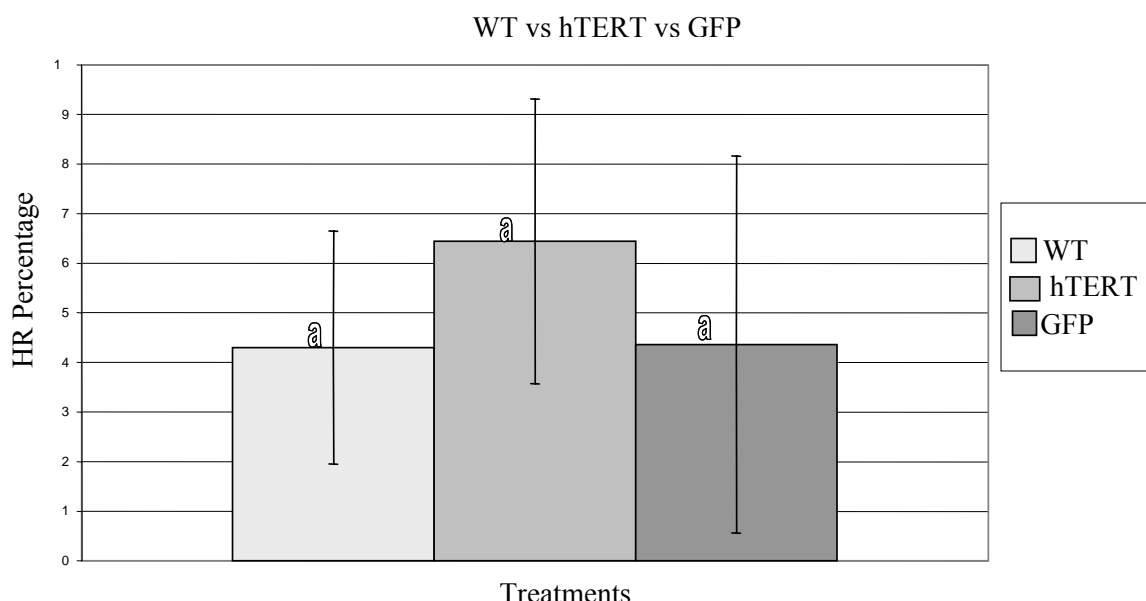


Figure 11. Percentage of colonies from HR of the two truncated plasmids in cells with or without telomerase. Error bars = standard error for each treatment group.

DISCUSSION

Among the many proteins involved in NHEJ, Ku 70/80, p53, DNA-PK, and the Rad50/Mre11/XRS2 complex have been studied extensively. Results indicated that these proteins as well as others are involved in many different telomeric functions (145). Among cell types shown to possess higher rates of HR there is a common aspect in that they all express telomerase. Examples of this are mouse ES cells, regenerating liver cells, and Fanconi's anemia cells (22-24). To date, there have been no reports on the relationship between the expression of telomerase and the rate of HR. To examine this relationship, PFFs virally co-transfected with hTERT and GFP and shown to express a functional hTERT, were compared to the same PFF either non-transformed or transfected with GFP alone. The use a plasmid to plasmid recombination system allowed for the direct comparison of the rates of HR between cells varying only in

telomerase expression. Unfortunately, no enhancement of HR was seen in the PFFs expressing hTERT. Additionally, hTERT was shown to be active via TRAP assay, however, no life extension was seen. This same cDNA has been shown to increase both life span and telomere lengths of human fibroblasts (146,147). However, studies by Zhu et al. (148) showed only life extension and no telomere elongation in previously transformed human fibroblasts. In addition, O'Hare et al. (149) found that transformations with hTERT alone was ineffective in immortalizing normal human mammary fibroblasts but required the addition of Simian virus 40 (SV40) large T antigen. While there have been no reports of hTERT expression in porcine cell lines, ectopic expression of hTERT has been reported in both sheep and bovine cell lines (150-154). However, only in sheep has hTERT been successfully expressed in normal fibroblasts (150,151). This hTERT expression was reported to have a dose dependent relationship on telomere length. While any level of expression of hTERT resulted in extended life span, only high levels resulted in telomere extension (150). Interestingly, it was found that while TRAP assay results showed only approximately two fold differences in the various hTERT positive cell lines, mRNA real time quantitative RT-PCR (qRT-PCR) and western blot analysis showed nearly 6000 fold differences indicating that the TRAP assay, while being able to pick up low levels of enzymatic activity, was not able to accurately quantitate the amount of hTERT mRNA and protein levels (150). This same group then attempted cloning with the high expressing hTERT positive sheep fibroblasts. These fibroblasts possessed normal karyotypes and telomere lengths and were able to develop to blastocyst stage at similar rates of control normal

fibroblasts. Transferred blastocysts also showed relatively high rates of implantation, early fetal development, and organogenesis. However, no fetuses survived past 40 days indicating the hTERT positive cells were not fully competent for nuclear transfer (151). Much is still unknown about the action of telomerase and the effects of ectopically expressing it in cells and, in the case of our PFFs, it is possible that hTERT is not able to activate the machinery needed to allow for extension of life span.

While Hata et al. (22) showed naturally hTERT expressing cells exhibited similar levels of HR as ES cells, Lindvall et al. (155) showed, by cDNA microarray, that ectopic expression of hTERT in normal human fibroblasts resulted in the up regulation of up to 172 genes and expressed sequence tags (ESTs). This suggests that hTERT transfection results in much more than simply hTERT expression. What is not known is whether it is the hTERT expression itself or the action of one or a multitude of other proteins activated by hTERT expression that may play a role in the similar levels of HR seen in telomerase expressing somatic cells as compared to ES cells. Because hTERT expression did not alter the life span of the PFFs one can not say if hTERT expression in these cells activated any of the other proteins seen in hTERT positive human fibroblasts. Therefore, the only conclusion that can be made from these experiments is that the expression of a functional hTERT in PFFs does not increase HR efficiencies. Once a porcine telomerase cDNA clone is isolated a more comprehensive study can be done to determine if porcine telomerase shows the same phenotypes in PFFs as human telomerase shows in human fibroblasts.

CHAPTER IV
EFFECTS OF CELL SYNCHRONY AND DNA NUCLEAR DELIVERY ON THE
RATES OF HOMOLOGOUS RECOMBINATION IN PORCINE FETAL
FIBROBLASTS

INTRODUCTION

When a cell encounters a DSB two unique pathways are available to repair it, either NHEJ or HR. Studies have shown that the NHEJ pathway tends to dominate in early S phase of the cell cycle while HR tends to dominate the repair in late S phase (9). Because NHEJ relies of the processing of the damaged ends of DNA to find the few compatible base pairs needed for ligation of the two ends, it is the most error prone of the two repair mechanisms. HR, on the other hand, requires large areas of homology such as sister chromatids, homologous chromosomes, and in the case of targeting, targeting constructs, to allow for strand exchange and invasion between the damaged and undamaged DNA. The repair of the DSB is then accomplished when the heteroduplex is resolved.

It has been known for quite some time that compounds that inhibit replication in mammalian cells are potent inducers of sister chromatid exchange, gene amplification, and HR (133-137). DSBs found when replication forks are disrupted are repaired by both NHEJ and HR although HR seems to be the dominant repair mechanism (138,156,157). Studies have shown that NHEJ may participate in an early response to

repair DSB while HR participates in a late response (138). Lundin et al. (139) showed that the two repair mechanisms occurred in cells arrested with hydroxyurea, which completely arrests the replication forks at the beginning of the S phase. However, when cells were arrested with thymidine, which only slows the replication fork, HR was the sole mechanism responsible for repairing the damaged DNA. While they showed that thymidine treatment did not cause detectable DSBs, they found that the defective HPRT gene was corrected only by HR in cells treated with thymidine.

Given this information, we investigated whether synchronization of cells at a point in the cell cycle, mid-late S phase, where HR is favored over NHEJ would increase the rate of HR.

Another method to further increase the rates of HR would be the more efficient delivery of the transgene to the nucleus, thus decreasing the time in the cytoplasm where it could be degraded. In addition, rapid delivery to the nucleus allows for longer exposure of the transgene to the HR machinery than if cell division and nuclear membrane breakdown is required. Studies of nuclear localization of cytoplasmic proteins have shown that they possess a nuclear localization signal (NLS) that interacts with specialized cytoplasmic proteins which transport the multimer to the nucleus via a nuclear pore complex (NPC) (131). In addition, Vacik et al. (132) has shown that plasmid DNA containing a 366bp sequence from simian virus 40 (SV40) origin of replication and early promoter were bound by cytoplasmic transcription factors that contain an NLS sequence, and were transported into the nucleus through the NPC. To test whether nuclear delivery of exogenous DNA is beneficially effected by the addition

of an NLS bearing protein recognition signal, an 180 base pair sequence from the early promoter region of the SV40 promoter of the pCDNA3 vector was added to the two truncated puromycin plasmids.

The work presented below was undertaken to determine the effects of cell synchronicity and efficient nuclear delivery of DNA had on the rates of HR in PFFs. A two plasmid recombination system using two truncated puromycin genes was used and the rates of HR in cells treated or untreated with thymidine with or without an NLS signal sequence present on the truncated plasmids were compared to normal cells treated or untreated with thymidine and transfected with a fully functional puromycin gene.

MATERIAL AND METHODS

Plasmids

Two truncated puro vectors were designed. Primers specific to either the pBluescript or puro gene were used to generate the plasmids using a pBS vector containing a pgk, puro, and bovine growth hormone polyA sequences for the PCR template (Table 1).

PCR conditions for the 3' Δ puro plasmid were initial denaturing at 94°C for 2 minutes, 30 cycles of denaturing at 94°C for 15 seconds, annealing at 62°C for 30 seconds, and elongation at 69°C for 2 minutes followed by a final elongation at 69°C for 7 minutes. The same conditions were used for the 5' Δ puro plasmid with the exception of the annealing temperature changed to 58°C. One μ l of Expand High Fidelity

polymerase mix, 20mM of each dNTP, and 1.5mM magnesium chloride was used. The resulting fragments were then cloned into a pCR2.1 vector.

The 3' Δ puro is a plasmid containing only the pgk promoter and a 3' end truncated puromycin acetyltransferase gene and lacks the poly A signal. The 5' Δ puro is a plasmid containing a 5' truncated puromycin gene and the poly A signal but lacks the pgk promoter. Both plasmids share approximately 580bp of homology in the puromycin gene. Neither plasmid is able to elicit puromycin resistance when transfected alone into a cell. However, when both plasmids are introduced into a cell, HR occurs via their shared homology and a fully functional puromycin resistant gene is formed (Fig. 6). Cells that have undergone a successful recombination event will be able to survive in the presence of puromycin. The rates of recombination, therefore, can be calculated by the number of puromycin resistant colonies that arise after selection.

The early promoter region of the SV40 promoter of the pCDNA3 vector (Invitrogen) was amplified using the following primers with FseI adapters at the 5' end (Table 4). The resulting 180bp PCR fragment was cloned into pCR2.1. This plasmid was then cut with FseI and cloned into the truncated plasmids via a synthetic FseI containing linker.

Table 4. PCR primers utilized for generating the NLS sequence

Primer name	Sequence (<u>FseI</u> site)
Early SV40 forward primer	<u>GGCCGGCC</u> GTGGAATGTGTGTCAGTTAG
Early SV40 reverse primer	<u>GGCCGGCC</u> ATGGTTGCTGACTAATTGAG

Fibroblast Cell Culture and Transfections

Purebred Duroc gilts on days 35 or 36 of pregnancy were hysterectomized and the fetuses removed. The head and viscera were removed from the fetus and the remaining tissue was minced with a sterile razor blade. The tissue was added to 10ml of 0.05% trypsin supplemented with 0.9mM potassium chloride, 0.9mM dextrose, 0.7mM sodium bicarbonate, 0.1mM EDTA, and 20mM sodium chloride. The tissue/trypsin solution was shaken at 37°C for 15 minutes a total of three times. After incubation, the supernatant was collected, pooled, and pelleted and expanded as described previously. The fibroblasts were then trypsinized and frozen in 50% FBS, 40% media, and 10% DMSO for long time storage and future use.

Fibroblast cells were re-established from freezing and grown in the (antibiotic free) media described above. The cells were expanded until appropriate numbers were obtained. At the time of electroporation, cells were trypsinized, counted, and aliquotted to 10×10^6 cells per treatment. Cells were pelleted and resuspended in 0.8ml of cold F10 Nutrient Mixture (HAM) and placed in a 4millimeter gap electroporation cuvette. DNA was delivered into the cells via electroporation (450 volts, 1 millisecond, 4 pulses).

In all experiments, 5nM of the linearized 3' truncated and of the circular 5' truncated plasmid was used. For the thymidine experiment, 24 hours prior to electroporation, 2mM of thymidine was added to the cells at 50-70% confluency. After each electroporation, cells were plated $1-2 \times 10^6$ cells per 10cm tissue culture plate and allowed to recover and attach overnight. Puromycin at the concentration of 2ug/ml was added to all plates approximately 24 hours after electroporation and the cells were grown

under selection until puromycin resistant colonies were visualized. Plates were washed with phosphate buffered saline and incubated in a 1:1 ratio of methanol:acetone at -20°C for 20 minutes, air dried, stained with trypan blue, and the colonies counted.

Flow Cytochemistry

To determine what percentage of cells were arrested in the S phase of the cell cycle, propidium iodide staining was performed following manufacture guidelines (DNAcon3-Consul^{T.S.}). Cells were analyzed on a FACSCalibur (Becton-Dickinson) flow cytometer, equipped with a 15mW air-cooled argon laser, using CellQuest (Becton Dickinson) acquisition software. Propidium iodide fluorescence was collected through a 585/42-nm bandpass filter and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI-width versus PI area. Data analysis was performed in ModFit LT (Verify Software House) using PI-width versus PI area to exclude cell aggregates.

Data Representation and Statistical Analysis

Data sets were transformed, when needed, to aid in acquiring normal distribution of values. Averages of six replications were subjected to ANOVA to determine differences between the means of the different treatments. If a difference was found, Fisher's Least Significant Difference analysis, using SPSS software, was performed to

determine which treatments were significantly different at the $p < 0.05$ level. Results are represented in average colony number of treatment groups (2 plasmids) divided by the average colony number of the positive control (single, fully functional puromycin resistance plasmid) multiplied by 100. This value represents the percentage of colonies that are the result of homologous recombination. The charts shown are the frequency of HR events per treatment as compared to the control.

RESULTS

Effects of Cell Synchrony on the Rates of HR

To study the effect of cell cycle on the rates of HR, cells were synchronized in late S phase by overnight incubation in 2mM thymidine. Propidium iodide staining and FACS was used to determine what stage of the cell cycle the cells were in prior to, during, and after thymidine treatment. As shown in Table 5, there was a five-fold increase in the percentage of cells in S phase after 24 hour incubation with thymidine. Following removal of thymidine the cells quickly recuperated and continued normally through the cell cycle.

Table 5. Cell cycle summary

Treatment	%in S phase	%earlyS	%midS	%lateS
Control	11.9	6.5	2.6	2.8
12hrs with Thymidine	53	40	10.6	2.3
24hrs with Thymidine	66	22.5	33.4	10.4
12hrs after remove Thymidine (normal Media)	35	7.3	2.7	25.4
24hrs after Thymidine	31.1	6.4	1.5	23.3
36hrs after Thymidine	22	4.7	2.6	14.7

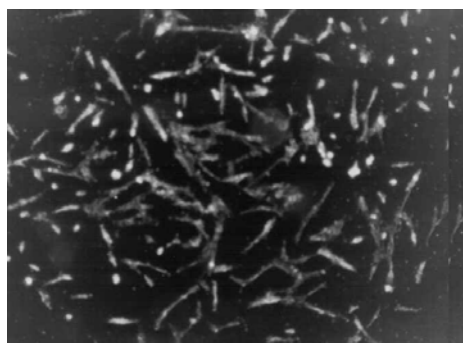
Percentage of cells in the given stages of the cell cycle during various time points before, during, and after thymidine treatment of normal porcine fetal fibroblasts

Growth Rates of PFFs Transfected with Plasmids with and without an NLS Signal

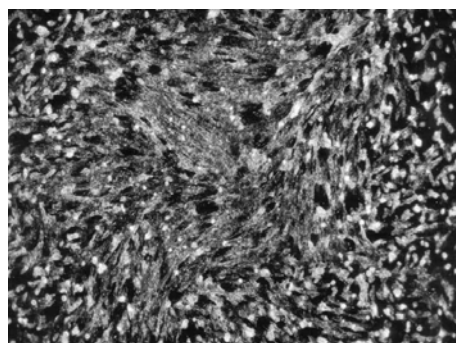
An interesting result was found when cells were transformed with the two truncated plasmids containing the NLS recognition sequence. When compared to the same cells transformed with the same plasmids without the NLS the NLS containing cells tended to be more robust. In addition, NLS cell colonies tended to be composed of more cells than that of non-NLS colonies. As summarized in Table 6 and Figure 12 totals from thirteen electroporations showed approximately two times more colonies in cells transfected with NLS containing plasmids. While there were both small and large colonies in each group, overall the colonies were healthier in the NLS cells.

Table 6. Summary from thirteen electroporations of colony number of NLS plasmid cells versus non-NLS plasmid cells

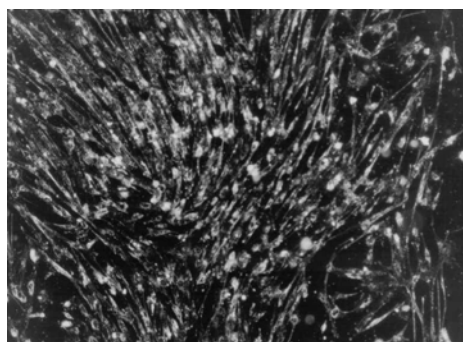
	Average Colony # Per Million Cells Electroporated \pm Standard Error	NLS Divided By Non-NLS Colony Number
NLS Cells	5.3 \pm 1.3	1.9
Non-NLS Cells	2.8 \pm 1.0	



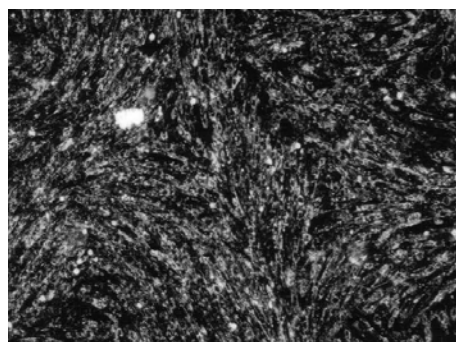
Non-NLS Plasmid Cell Colony



NLS Plasmid Cell Colony



Non-NLS Plasmid Cell Colony



NLS Plasmid Cell Colony

Figure 12. Representative pictures of NLS plasmid containing cells and Non-NLS plasmid containing cells.

Effects of Nuclear DNA Delivery on the Rates of HR and NHEJ

To determine what the effect of faster delivery of the transgene to the nucleus would have on HR, an NLS-bearing-protein recognition signal was added to the two plasmid recombination system. Additionally, the interaction between cell synchrony and the use of NLS was tested. The average of six experiments using day 35 and 36 old fetal fibroblast cell lines show significant differences ($p < 0.05$) in the percentage of HR: the control plasmids (truncated plasmids with no NLS) with and without thymidine treatment, the control plasmids without thymidine and the NLS containing plasmids with thymidine, the control plasmids with thymidine and the NLS containing plasmids without thymidine, and the NLS plasmids with and without thymidine (Fig. 13).

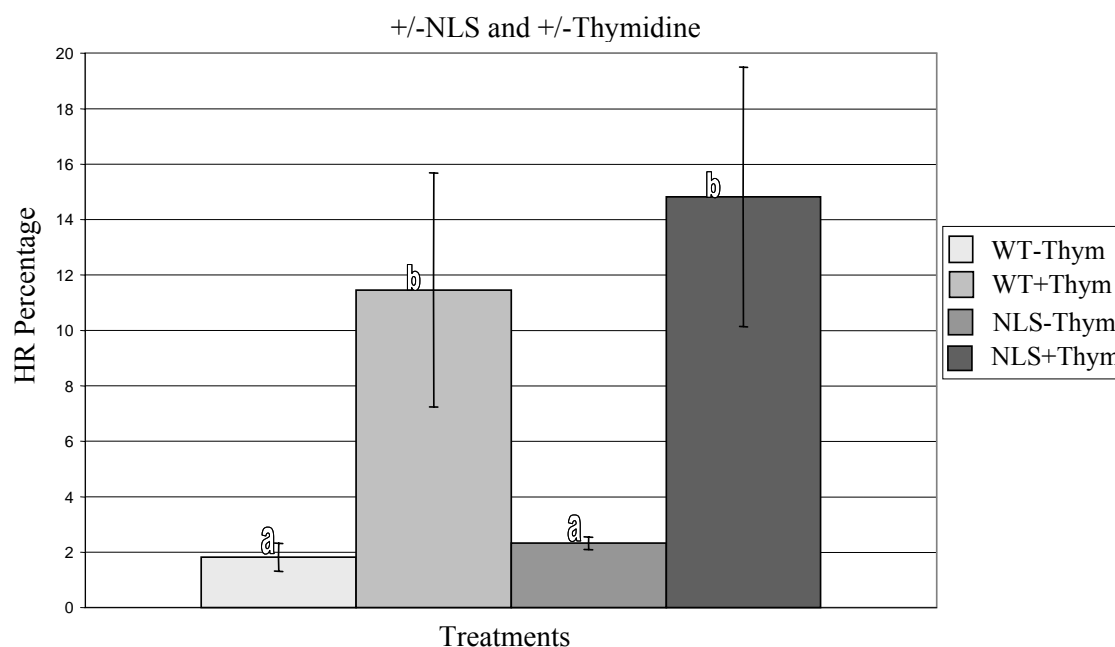


Figure 13. Percentage of colonies resulting from HR of the two truncated plasmids either with or without the NLS binding sequence and with or without thymidine. Error bar = standard error of each treatment group. ($p < 0.05$)

DISCUSSION

An alternative method to enhance HR would be to have the majority of the cells synchronized at a point in the cell cycle, mid-late S phase, where HR is favored over NHEJ. Excess thymidine in culture has been shown to inhibit the formation of dCTP, an essential precursor to DNA, and therefore dramatically slow the replication fork at the latter part of the S phase of the cell cycle (158). Studies by Lundin et al. (139) indicate that after thymidine incubation, correction of the HPRT gene in Chinese hamster cell lines was exclusively the result of HR and not NHEJ. However, a direct comparison of HR rates between normal and thymidine blocked cells has not been reported. Our results show a significant increase in the rates of HR between all plasmids involving thymidine incubation as compared to the same plasmids without thymidine. This increase in HR is likely due to an increase in Rad51 and other HR molecules present in the cells during late S phase and as a result of thymidine treatment. This theory is further supported by the work of Lundin et al. (139) who found a 24-fold increase in HPRT⁺ recombinants (presumably due to HR) and an increase in Rad51 foci in cells treated with thymidine.

In addition to having cells in the proper stage of the cell cycle, more efficient delivery of the transgene to the nucleus should have a positive effect on the rates of HR. Decreased time in the cytoplasm where the exogenous DNA could be degraded and rapid delivery to the nucleus allowing for longer exposure of the transgene to the HR machinery would both contribute to the higher rates. Vacik et al. (132) have shown that plasmid DNA containing a 366bp sequence from SV40 origin of replication and early

promoter were bound by cytoplasmic transcription factors that contain an NLS sequence, and were transported into the nucleus through the NPC. The 180 bp sequence containing a 72bp tandem repeat found in the early SV40 promoter was inserted into both of the truncated plasmids. Comparison of these plasmids to plasmids without the added sequence showed an increase in the rate of HR.

Results indicate an additive effect of both thymidine synchronization and the addition of an NLS-bearing-protein binding site on the rates of HR. The further enhancement of HR by the NLS can be seen by the slight non-significant increase in HR with the NLS bearing plasmids with thymidine as compared to the control plasmids with thymidine. In addition, separate experiments comparing only NLS and non-NLS plasmids showed a two fold increase in colony numbers from cells containing the NLS plasmid.

Traditional targeting strategies include transfecting asynchronous cell population (20% to 30% of cells in S phase at any given time). Non-S phase transfected cells must go through the cell cycle to re-enter S phase and during this time the transgene is in the presence of enzymes present in the cytoplasm. Synchronizing the cells with thymidine (greater than 60% of cells in S phase) allows for a much greater percentage of cells ready for HR to occur. The addition of the NLS signal on the plasmids may further enhance the rates of HR by transporting the transgene into the protective environment of the nucleus faster where HR can take place.

Recombination can take place between three types of substrates: extra-, intra-, and inter-chromosomally (for reviews see 84,159). Extra-chromosomal recombination

involves two DNA sequences neither of which are associated with chromosomes (i.e. two independent plasmids). Intra-chromosomal recombination involves the recombination of two sequences located on the same DNA molecule (i.e. tandem plasmid of two sequences or the two sequences on the same chromosome). Inter-chromosomal recombination is the recombination of two sequences on two separate chromosomes or between a chromosome and a targeting plasmid. Chromosomal DNA is bound by histones and other regulatory proteins as well as having areas of heterochromatin, all of which can hinder its accessibility to HR and NHEJ molecules. Extra-chromosomal sequences on the other hand are not impeded by these obstacles. Because of this, some reports have suggested that results from plasmid to plasmid assay experiments may not necessarily reflect what would occur in normal inter-chromosomal recombination (18,84,159-161).

Extra chromosomal recombination (ECR) in mammalian cells has been used to study recombination mechanisms for over 20 years (for review 159). This method has been chosen mainly due to the ability to obtain results rapidly and the ease of which to manipulate the systems. One of the most used protocols involves the use of two non-functional selectable markers (usually a neomycin resistant gene) transfected either in two individual plasmids or in tandem on a single plasmid. Selection media is added 24 hours after transfection and colonies of resistant cells are visible approximately two weeks later. Recombination frequency is calculated by dividing the number of resistant cells by the number of colonies arising from a wild type, functional marker transfection. Because selection occurs over a two week period, incorporation of the functional

selectable marker must occur. ECR frequencies have been reported in the range of 1-20% (159,162-167) and the frequency of ECR involving integration has been shown to be equal to or greater than those obtained from systems that do not involve integration (168). Based on observations that ECR correction and integration occurred 10 times more frequently than integration of the wild type marker, Wong and Capecchi (168) have suggested that ECR is coupled with the subsequent integration of the recombined molecule. If the two events are indeed linked then the use of this system to infer on other types of recombination may lead to an over-estimation of HR frequency. In addition, there have been variable reports on when in the cell cycle ECR takes place (159,162,169,170), the extent of homology between the two sequences necessary for efficient HR (159,167,171,172), and what recombination mechanism is responsible for ECR (for review see 159). The only safe conclusion to be made in this field is that ECR in mammalian cells involves a multitude of pathways and varies from one experiment to another thus making a definitive conclusion difficult. However, due to the difficulties in studying HR in normal mammalian cells using traditional targeting vectors the use of ECR is the most practical way of studying the process.

Until the exact mechanisms of HR in somatic cells is elucidated or methods are developed that can extend the life span while retaining the cells ability to be efficiently cloned, any protocol that can increase the efficiency of HR is useful. Because we used a plasmid to plasmid recombination system in this study the increases in HR may not reflect actual HR rates in somatic cells between a chromosomal gene and an extra-chromosomal transgene but the trends should follow that seen in our system. Due to the

current inefficiencies of targeting livestock somatic cells, all labs have relied on the promoter trap for enrichment. With the development of better protocols it could now be possible to target non-expressing genes thus opening a vast area of research previously inaccessible until now.

CHAPTER V
EFFECTS OF ENHANCERS ON TARGETING OF THE ALPHA 1,3
GALACTOSYLTRANSFERASE (GT) GENE

INTRODUCTION

The GT gene product is an enzyme responsible for the addition of a galactose- α 1,3-galactose carbohydrate epitope onto the cell surface (173). The GT gene has been inactivated through several mutations in humans, apes, and old world monkeys. In addition, through continued exposure to gastrointestinal bacteria that express similar carbohydrate antigens, humans produce large amounts of anti-Gal antibodies (estimated to be as much as 1% of the total circulating Ig) (174,175). Because of the presence of these antigens on pig cells, and the presence of the naturally occurring antibodies in humans, the introduction of human blood to a pig organ quickly results in a hyperacute immune response (HAR) (173,176-178). Many non-transgenic and dominant negative/over-expression transgenic strategies have been attempted to by-pass human HAR to pig organs such as the temporary depletion of the anti-Gal antibodies before transplantation, the treatment of pig organs with α -galactosidase to remove the surface epitopes, the over expression of competing transferases to lessen the levels of GT expression, and the expression of human complement inhibitors such as human decay-acceleration factor and human CD59 (173,179-181). However, none of these strategies worked in eliminating all of the epitope expression and the residual α -1,3 Gal epitopes

were sufficient to activate the HAR. Complete removal of the epitopes through the complete inactivation of the GT gene via homologous recombination should therefore cause a complete blockage of the HAR. Human blood perfusion studies performed on the GT double knockout mouse has shown substantially less antibody binding resulting in a significant decrease in complement activation and HAR (182). Three groups (71-73) have successfully inactivated one copy of the pig GT gene and one of these groups recently succeeded in isolating and cloning GT double knockout pigs (75). However, no studies involving human blood perfusion have been reported using any of these animal's tissues.

There are basically three types of HR: single strand annealing (SSA), gene conversion (GC), and break-induced replication (BIR) (183). SSA is the simplest of the mechanisms and involves the resection (5' to 3') and exposure of complementary regions of homology sequences originally flanking the DSB. This process is prone to creating deletions and will occur with as little as 30bp of homology although it is more efficient with 200-400bp (184). Gene conversion is in competition with SSA in that GC uses the two resected ends to invade and copy the sequences from a homologous template located on a sister chromatid, homologous chromosome, or an ectopic location (i.e. targeting construct). Because of this, GC is considered the least error prone of the HR mechanisms. The third type of HR is break induced recombination. BIR occurs when only one resected end of the DSB participates in the exchange with homologous sequences. Elongation of this single invading strand off of its homologous sequence will continue until it either meets the end of the chromosome or until it encounters a

converging replication fork. Only GC and BIR involve invasion and elongation and therefore involve the action of Rad51 while SSA is Rad51 independent.

Due to the availability of the three types of recombination mentioned above and because of the possible discrepancy between extra- and inter- chromosomal recombination discussed in the previous chapter, testing of some of the enhancers presented previously were tested on an actual locus, the α -1,3-galactosyltransferase (GT) gene, using several different types of targeting vectors. From the previous results we determined that mutated p53, thymidine synchronization, and the addition of an NLS-bearing protein recognition signal positively affected the rate of plasmid to plasmid recombination. However, because of the tendency of cells with mutant p53 to become aneuploid there was concern in using this type of enhancement in cells destined to be cloned. While the use of both thymidine synchronization and NLS seem the best choices for enhancing HR in PFFs, most of the enhancers discussed in the previous chapters were examined to some extent.

MATERIAL AND METHODS

Generation of GT Targeting Constructs

Using the porcine α 1,3 galactosyltransferase cDNA sequence obtained from the GenBank (NCBI) database, the primers were designed (Table 7). Long range PCR (LR-PCR) on porcine genomic DNA using primer set GT1A resulted in a six kilobase fragment and primer set GT1B resulted in a seven kilobase fragment (Fig. 14). The two

fragments were then inserted into pCR2.1 vectors using the TA cloning technique (Invitrogen). The two plasmids were sequenced and confirmed via Blast search (GenBank) as containing porcine GT exon coding sequences (Fig. 14).

Table 7. PCR primers utilized for generating the two GT constructs

Primer name	Sequence
GT1A forward primer	ATGGAAGGCTCCAGTGGTATGG
GT1A reverse primer	GACCTGATCCACGTCCATGCAG
GT1B forward primer	AAAGAAGACAACAGAGGAGAGCTTCCGC
GT1B reverse primer	GTGCCTTCCCATAACCACTGGAGCC
GT2 forward primer	AGAGGTCGTGACCATAACCAGAT
GT2 reverse primer	AGCCCATCGTGCTGAACATCAAGTC

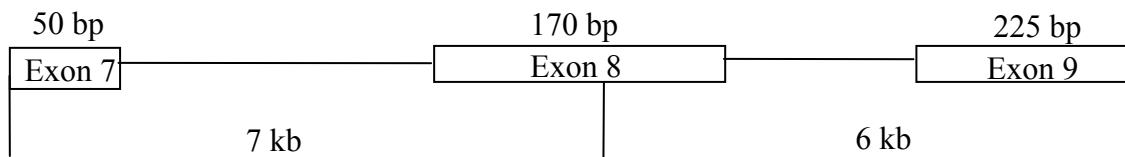


Figure 14. Schematic of the DNA amplified by long range PCR using primers specific to GT.

Using these two plasmids as the basis for the two “arms” of the final construct, various restriction enzymes were used to obtain a five kilobase fragment from the

original seven kilobase fragment for insertion into pBS. Using this plasmid, the pgk, puromycin acetyltransferase, poly A gene (minus either the promoter or poly A part of the gene) was inserted. Inserting a 4kb fragment from the original 6kb PCR fragment downstream from the puromycin-resistant gene completed the left arm of the targeting construct (Fig. 15).

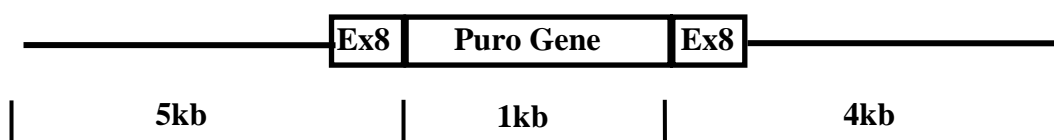


Figure 15. Schematic of the first completed targeting construct used to target the GT gene in porcine fibroblasts.

An additional plasmid similar to the targeting construct used by Dai et al. (72) to target the GT locus was constructed. LR-PCR was performed on porcine genomic DNA using the GT2 forward and reverse primers (Table 7). The resulting 7.4kb fragment included most of exon 8 through exon 9 of the GT gene. A 1.8kb internal ribosome entry site (IRES), neomycin poly A fragment was then inserted into exon 9 to disrupt gene function and allow for the selection of targeted events (Fig. 16).

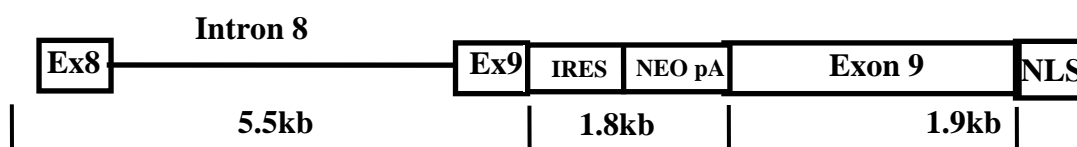


Figure 16. Schematic of the second completed targeting construct used to target the GT gene in porcine fibroblasts.

The early promoter region of the SV40 promoter of the pCDNA3 vector (Invitrogen) was amplified using primers with FseI adapters at the 5' end (Table 4). The resulting 180bp PCR fragment was cloned into pCR2.1. This plasmid was then cut with FseI and cloned into both GT plasmids via a synthetic FseI containing linker.

Isolation of Fibroblast Cells

Purebred Duroc gilts on days 35, 36, or 57 of pregnancy were hysterectomized and the fetuses removed. For day 35 and 36 fetuses, the head and viscera were removed and the remaining tissue was minced with a sterile razor blade while with the day 57 fetuses, the skin was removed from the hind leg and the muscle was minced. The tissue was added to 10ml of 0.05% trypsin supplemented with 0.9mM potassium chloride, 0.9mM dextrose, 0.7mM sodium bicarbonate, 0.1mM EDTA, and 20mM sodium chloride. The tissue/trypsin solution was shaken at 37°C for 15 minutes a total of three times. After incubation, the supernatant was collected, pooled, and pelleted. The cell pellet was resuspended in DMEM/F12 media supplemented with 10%FBS and 5%CS, 30mM sodium bicarbonate, 0.5mM pyruvic acid, and 2mM N-acetyl-L-cysteine. In addition, 100U penicillin, 100ug, and 250ng amphotericin were added to inhibit microbe growth. The fibroblast cells were placed in the appropriate number of 10cm tissue culture plates and placed in a 5% CO₂ incubator at 39°C and allowed to attach and grow to confluency and then passed (1:2 or 3). The cells were then trypsinized and frozen in 50% FBS, 40% media, and 10% DMSO.

Electroporation

Cells were re-established from freezing and grown in the (antibiotic free) medium described above. The cells were expanded until appropriate numbers were obtained. At the time of electroporation, cells were trypsinized, counted, and aliquotted to 10×10^6 cells per treatment. Cells were pelleted and resuspended in 0.8ml of cold F10 Nutrient Mixture (HAM) and placed in a 4millimeter gap electroporation cuvette. DNA was delivered into the cells via electroporation (450 volts, 1 millisecond, 4 pulses). Approximately 10×10^6 cells were electroporated with 5nm of either the linear or circular construct with or without 5nM of the enhancers (i.e. truncated Mre11, Anti-Ku RNA aptamer, mutated p53, or the addition of the NLS signal to the targeting plasmid). Another 2×10^6 cells were electroporated with either 5nM of the pgk puro pA DNA control or with no DNA as a negative control. For the thymidine experiments, 24 hours prior to electroporation, 2mM of thymidine was added to the cells at 50-70% confluency. After electroporation the cells were given time to recover and then put on selection when 70% confluency was reached. Selection with 1.5 to 2.0 ug/ml of puromycin was carried out for about ten days or until a visible, compact colony had grown. Individual colonies were then passed via cloning rings into a well of a 96-well plate. After several rounds of passages up to at least two wells in a 24 well plate one half of the cells were frozen in liquid nitrogen in a 50% serum, 10% DMSO media solution while the second half was used for DNA or RNA extraction following standard protocol.

Lipofection

As an alternative to electroporation, lipofection, was also studied. Fibroblasts were transfected using LipofectAMINE[™] 2000 Reagent (Life Technologies). Following the suggested protocol, the appropriate amount of DNA (either linear or circular construct with the appropriate controls) plus 5nM of enhancer molecules (i.e. truncated Mre11, Anti-Ku RNA aptamer, mutated p53) were added to the lipofection solution and put on the fibroblast overnight. After sufficient time for recovery, the cells were put on selection (1.5 to 2.0 ug/ml puromycin) for about 10 days or until a visible, compact colony had grown. The cells were then passed and analyzed as in the electroporation protocol.

Diagnostic Testing for Possible Positive Targeting Events

The integrity of the extracted DNA and RNA was checked on a 0.8% gel. Approximately 50-100 ng of DNA was used in each subsequent PCR. For GT1, several primer pairs were used in the PCR diagnostic procedures. One half of these primer pairs contain one primer to different endogenous areas of the targeted gene and the other primer to areas of genomic DNA inside the construct. The second half of primer pairs contains one primer to different endogenous areas of the targeted gene and the other primer to the puromycin resistance gene inside the construct (Fig. 17). Analysis of several PCR results by Southern blot using both genomic DNA and puromycin DNA as probes was used to determine which puromycin resistant colonies were true targeted events. In addition, in certain experiments, 7.5ug of RNA was used for first strand

cDNA synthesis following the Prostar First Strand RT-PCR Kit protocol. The primers used to amplify regions of the targeted cDNA are presented in Table 8 and a schematic of these areas on the cDNA is presented in Fig 18.

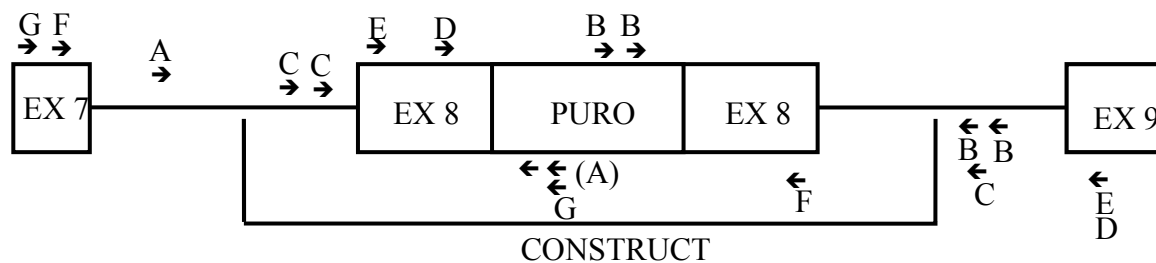
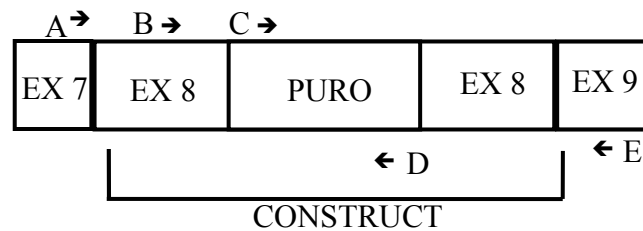


Figure 17. Schematic of primers used for the diagnostic tests on possible positively targeted GT1 colonies. Like letters represent primer pairs used for LR-PCR. When the same letters are together indicates nested or semi-nested primer sets. The area of genomic DNA encompassed by the targeting construct is indicated.

Table 8. PCR primers utilized for generating RT-PCR product from possibly GT1 targeted colonies

Primer name	Sequence
(A) GT-Exon7 forward primer	AAAGAAGACAACAGAGGAGAGCTTCCGC
(B) GT-Exon8 forward primer	AAACGCCCAGAGGTCGTGAC
(C) Puro forward primer	CCGAGTACAAGCCCACGG
(D) Puro reverse primer	GCTTGCGGGTCATGCAC
(E) GT-Exon9 reverse primer	GAACTCCTCCAAGTAATGCTCAATG

**Figure 18.** Schematic of the priming sites of the cDNA diagnostic primers. A,B, and C are forward primers while D and E are reverse primers. The area of the cDNA encompassed by the construct is indicated.

To determine positive targeting events using the GT2 plasmid the primers listed in Table 9 and shown schematically in Fig. 19 were used on genomic DNA isolated from G418 resistant colonies. All colonies that survived transfer, passage, and grew well enough to have DNA extracted from them were analyzed by LR-PCR using primer set B/D, transferred to membranes, and probed with the IRES probe. Possible positives from this initial screen were re-probed with a neo probe and were also further analyzed

by LR-PCR using primer sets A/E and C/F. Membranes with primer set C/F LR-PCR products were then probed with the IRES probe.

Table 9. PCR primers utilized for generating LR-PCR product from possibly GT2 targeted colonies

Primer name	Sequence
(A) GT2-Intron7 forward primer1	TGTCCTCCCCCAGAAGATG
(B) GT2-Intron7 forward primer2	CCTTAGCAAAGCCATCCTGACTC
(C) GT2-Intron7 forward primer3	TTAGCAAAGCCATCCTGACTC
(D) GT2-Exon9 reverse primer	AGCCCATCGTGCTGAACATCAAGTC
(E) GT2-Neo reverse primer2	GGATACTTTCTCGGCAGGAGCA
(F) GT2-IRES reverse primer1	GTTGAATACGCTTGAGGAGAGC

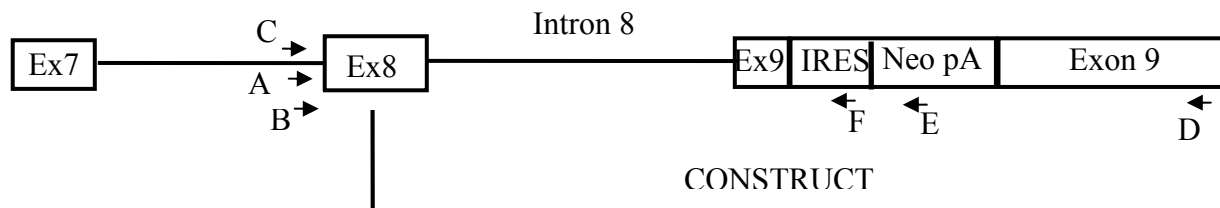


Figure 19. Schematic of the priming sites for the diagnostic primers used in finding possible positively targeted colonies using the GT2 construct. Primers A, B, and C are forward primers while primers D, E, and F are reverse primers. The area of genomic DNA encompassed by the construct is indicated.

Southern Blot Analysis

PCR products were visualized by electrophoresis through a 0.8% agarose gel in 1xTAE. The gel was stained with ethidium bromide to visualize and document the PCR product and DNA size ladder. The gel was soaked for 1 hour in 50mM NaOH and 1.5M NaCl to denature the DNA and then transferred to 500mM Tris-HCl (pH 7.4) and 1.5M NaCl for 30 minutes to neutralize before soaking in 20xSSC (3M NaCl, 300mM Na₃Ci). DNA was transferred to nitrocellulose by capillary action of 20xSSC through filter paper and DNA was cross linked to the membrane by UV exposure for 3 minutes and dried at 80°C for 2 hours.

The four probes used, puro probe, IRES probe, neo probe, and GT exon 8 probe, were generated as follows. The puro probe primers listed in Table 10 were used to generate a 327bp fragment of the puromycin gene which was then cloned into a pCR2.1 vector. The fragment was excised from the plasmid with EcoRI, gel purified and used for probing. The exon 8 probe was generated from an ApaI and EcoRI digest of the PCR product of the GT1A primer set (Table 7 and Fig. 14) resulting in a 200bp piece that was sub-cloned into pBS. The fragment was excised from the plasmid using ApaI and EcoRI, gel purified, and used for probing. The IRES probe was generated from HindIII digest of the GT2 targeting construct (Fig. 16). The resulting 348bp fragment represented part of the IRES and was sub-cloned into pBS. The fragment was excised from the plasmid with HindIII, gel purified, and used for probing. The second probe used in the GT2 diagnostics was the neo probe. This probe was generated by BamHI

and PvuII digest of the pBSNEOTK plasmid. The resulting 560bp neomycin gene fragment was gel purified and used for probing.

Table 10. PCR primers utilized for generating the puro probe used in Southern blot

Primer name	Sequence
Puro Probe forward primer	CGAGTACAAGCCCACGGTGC
Puro Probe reverse primer	GCCTTCCATCTGTTGCTGCG

Radioactive probe (^{32}P -dCTP, Amersham) was random primed using the NEBlot Kit (New England Biolabs) according to manufacturer's recommendations and purified through a Sephadex G-50 column. Blots were hybridized with Rapid Hyb (Amersham) and 1 million dpm/ml of hybridization solution at 65°C for 2 hours. Hybridization solution was removed and the blots were washed at room temperature with either 4xSSC with 0.1% SDS for 10 minutes or a series of washes (2xSSC at room temperature, 0.1xSSC twice at 65°C) with 0.1% SDS for 15-20 minutes before exposure to radiography film.

Cloning

Oocytes, obtained from Bomed (Madison, WI), were matured in Earle's TC199-Hepes supplemented with 5ug/ml insulin, 10ng/ml EGF, 0.6mM cysteine, 0.2mM sodium pyruvate, 25ug/ml FSH, and 10% porcine follicular fluid for 40 hours prior to manipulation (all chemicals were purchased from Sigma unless otherwise noted).

Following 38-40 hours of maturation, cumulus cells were removed from the oocyte by vortexing in 0.1% hyaluronidase in TC199. Oocytes were then rinsed through three drops of manipulation media (salt buffered Ca^{+} free NCSU-23 containing 5% FCS, osmolality 297 ± 3 with pH 7.4). The manipulation media was made by replacing sodium bicarbonate with enough sodium chloride to increase osmolality to 297 ± 3 , and the pH was adjusted to 7.4 with 1M sodium hydroxide. The oocytes were incubated in manipulation medium containing 5ug/ml cytochalasin B, and metaphase II oocytes were enucleated by removal of the first polar body and metaphase plate utilizing a 25um beveled glass pipette. Absence of the metaphase plate was visualized by exposure to ultraviolet florescent light.

Reconstruction was conducted in manipulation media. Porcine fetal fibroblasts were seeded in a 4 well plate at 35% confluency and grown until contact inhibited. The cells were trypsinized until the cells started to detach and were then resuspended in salt buffered NCSU-23 containing 10% FBS (185). The cells were placed in a separate drop of salt buffered NCSU-23 with 0.8mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 10%FBS. Groups of 15-20 cells were loaded in the pipette and a single cell was placed in the perivitelline space of the oocyte. Following reconstruction, the oocytes were placed in a 1mm fusion chamber and given an alignment pulse of 2 volts alternating current (AC) for 2 seconds followed by 2 direct current (DC) pulses of 40 volts for 50 microseconds in 297mM Mannitol, 0.001mM CaCl_2 and 0.05mM MgCl_2 . Following fusion, the oocytes were placed in bicarbonate buffered NCSU-23, osmolality 297 ± 3 with 10% FBS for 1 hour prior to activation.

Reconstituted oocytes were activated by two DC pulses of 120 volts for 60 microseconds in 297mM Mannitol, 0.1mM CaCl₂, and 0.05mM MgCl₂. Following activation the oocytes were placed back in bicarbonate buffered NCSU-23 osmolarity 297 \pm 3 with 10% FBS for 1-3 hours until transfer.

All reconstructed oocytes were transferred into naturally cycling gilts on the first day of standing estrus. A mid-ventral laparotomy was performed, the reproductive tract was exposed and the reconstructed embryos were transferred into the oviduct at the ampullary-isthmus junction. All experiments were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Maintenance of pregnancy was aided by the forced ovulation/corpus luteum development using a combination of PMSG and hCG. PMSG (1,250 IU) was injected i.m. on day 11 of the estrous cycle (day 0 = first day of standing heat) and 750 IU of hCG was injected i.m. three days later (day 14 of the cycle).

RESULTS

Electroporation and Lipofection of the GT1 Targeting Plasmid

As summarized in Table 11, over one hundred electroporations using either the promoter trap construct (~60) or the polyA trap construct (~41) and approximately twelve lipofections using either the promoter trap construct (~5) or the polyA trap construct (~7) were performed and the colonies surviving puromycin selection analyzed. A total of 131 colonies survived selection and 68 (52%) of these survived passages and

grew well enough for DNA and/or RNA isolation. While there is not enough data to determine a statistical difference, there was a marked difference between lipofection and electroporation in both the number of colonies and the number of colonies surviving passages. There was a 34 fold increase in the number of circular polyA trap colonies and a 9.4 fold increase in linear promoter trap colonies using lipofection instead of electroporation for the introduction of the construct into the cell. Also, 100% of the lipofection colonies surviving selection survived the passages while only 37% of the electroporated colonies survived.

Table 11. Summary of electroporations and lipofections

Linear/Circular Construct	Electroporation/Lipofection	Number Of Cells Used	Col. Surv. Trt.	Col. Surv. Pass.	Col. PCR	Pos. Col.	Enrich With Anti Ku + p53	Enrich With Anti Ku	Enrich With p53
Circular PolyA Trap	Electroporation	230x10 ⁶	28	13	13	0	0	0	0
Circular PolyA Trap	Lipofection	10x10 ⁶	4	4	4	0	1	1	1
Circular Promoter Trap	Electroporation	80x10 ⁶	0	0	0	0	0	0	0
Circular Promoter Trap	Lipofection	10x10 ⁶	0	0	0	0	0	0	0
Linear PolyA Trap	Electroporation	175x10 ⁶	22	13	13	0	1	0	0
Linear PolyA Trap	Lipofection	6x10 ⁶	0	0	0	0	0	0	0
Linear Promoter Trap	Electroporation	520x10 ⁶	50	11	11	0	0	0	0
Linear Promoter Trap	Lipofection	42x10 ⁶	27	27	27	1*	6	2	1

* See below

Summary of electroporations and lipofections performed using the GT1 targeting plasmid either with a promoter trap or polyA trap and either linearized or circular constructs. In some experiments, various enhancers were added and the colonies surviving in this treatment are also presented. Data presented include the total number of cells transfected, the number of colonies surviving the initial selection, the number surviving the expansion (passage), the number of colonies analyzed by PCR and those found positive for a targeting event, and the number of colonies that resulted from experiments involving the addition of the indicated enhancers.

DNA was extracted from all the surviving colonies and a series of primers were used for LR-PCR analysis followed, in most cases, by Southern blot analysis of the LR-PCR products using either a puro probe or an exon 8 probe. No colony showed conclusive results indicating a positive targeting event (Table 12 and Fig. 20).

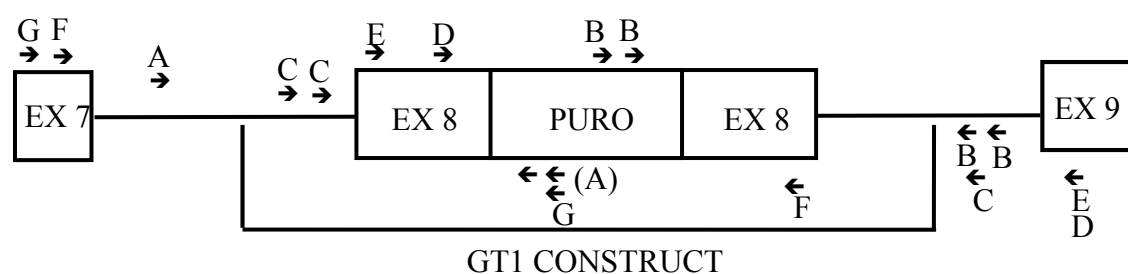


Figure 20. Schematic of primers used for diagnostic analysis of the genomic DNA using LR PCR on GT1 colonies surviving puromycin selection. Like letters represent primer pairs used for LR-PCR. When the same letters are together indicates nested or semi-nested primer sets. The area of genomic DNA encompassed by the targeting construct is indicated.

Table 12. PCR primers utilized for diagnostic tests

Primer Set (Type)	Number of colonies analyzed	Expected Bands if Targeted	PCR Results	Southern Blot Results (Probe)
A (hemi-nested)	5	1	No correct size bands	N/A
B (nested)	6	1	No correct size bands	Non-specific binding (puro)
C (hemi-nested)	0	2	Could not optimize No correct size bands	N/A
D (normal)	15	2	Correct size bands	Non-specific binding
E (normal)	16	2	Single band only	N/A
F (normal)	60	2	Multiple bands some Include correct size	Non-specific binding (puro) Binding only to lower/endogenous band (exon8)
G (normal)	42	2	Multiple findings: No bands, Single band on some electroporations, Two bands on some Lipofections	Non-specific binding (puro) Binding only to lower/endogenous band (exon8)

PCR primers utilized for diagnostic tests on the surviving colonies of the GT1 construct transfections. Primer name, number of colonies analyzed, expected LR-PCR results, and actual LR-PCR and Southern blot results are given.

A positive but inconclusive result from a LR-PCR using primer set E on one colony from a lipofection using linearized promoter trap GT1 with the addition of the anti-Ku RNA aptamer, mentioned in chapter II, was determined promising and was

chosen for further analysis. Total RNA was isolated from this colony and RT-PCR was performed using the primers in Table 8. The ethidium bromide gel of the PCR products is shown in Fig. 21.

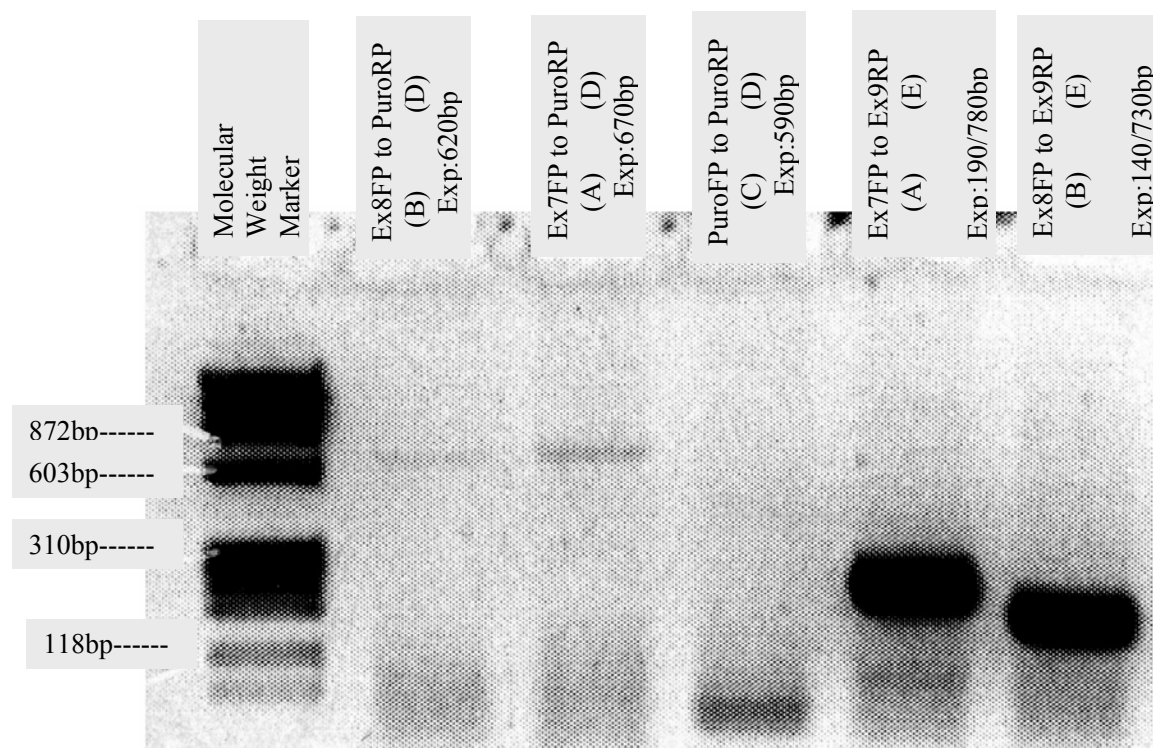


Figure 21. Ethidium bromide gel showing results from RT-PCR of D883-3 PFF lipofected with linear GT1 promoter trap DNA and anti-Ku RNA aptamer. Primer set B/D resulted in the expected 620bp fragment, primer set A/D resulted in the expected 670bp fragment, primer set C/D did not result in the expected 590bp fragment. Primer sets A/E and B/E resulted in the smaller expected fragment (190bp/140bp respectively), but not in the larger expected fragment (780bp/730bp respectively).

Due to the nature of the primers used for RT-PCR, a correctly targeted colony would result in a 670bp fragment from primer set, ex7FP to puroRP, and a 620bp fragment from the primer set, ex8FP to puroRP. The non-targeted second allele would

not give a PCR result because of the puro binding site of the reverse primer. Similarly, the puro forward and reverse primers should only give a result from the targeted (or randomly inserted) gene, however, we did not see a band at the expected size of 590bp. The primer sets, ex7FP to ex9RP and ex8FP to ex9RP, should amplify two fragments, one from the non-targeted allele (190bp/140bp respectively) and the other from the targeted allele (780bp/730bp respectively). However, only the smaller endogenous band was visible after PCR probably due to the preferential amplification of the smaller product, a phenomenon common in PCR.

Because of the promising results of the presence of a correct size band in both the ex7FP to puroRP and ex8FP to puroRP, it was decided to attempt cloning of these cells in order to first, determine if it would be possible to clone PFFs that had been grown for over 40 days and had undergone strenuous selection and secondly, to obtain enough tissue from the cloned piglets to do a genomic Southern blot, a process that is hindered in cell culture due to the finite lifespan of the PFFs. Cloning resulted in the birth of eight healthy, normal Duroc piglets and one diminutive Duroc piglet that died three days after birth (Fig. 22). The remaining eight piglets continued to be healthy and developed normally until the time of their slaughter four and one half months later. Genomic DNA was isolated from ear and tail samples from all piglets. However, Southern blot analysis using either the exon 8 probe or the puro probe yielded ambiguous results (data not shown). LR-PCR using primers from exon 7 to the 3' end of exon 8 resulted only in the endogenous non-targeted band. RNA was then isolated and RT-PCR with various primers were used to determine if the GT allele was indeed targeted. Unfortunately, no

RT-PCR results showed conclusive proof of gene targeting. In order to determine whether these clones were transgenic, a puro forward and reverse primer was use for PCR on genomic DNA from all piglets. The product was gel purified, cloned into pCR2.1 and sequenced. Sequence analysis showed the existence of a normal puromycin gene fragment expected given the primers used. This proves that while the GT allele may not have been targeted, viable transgenic piglets were produced.

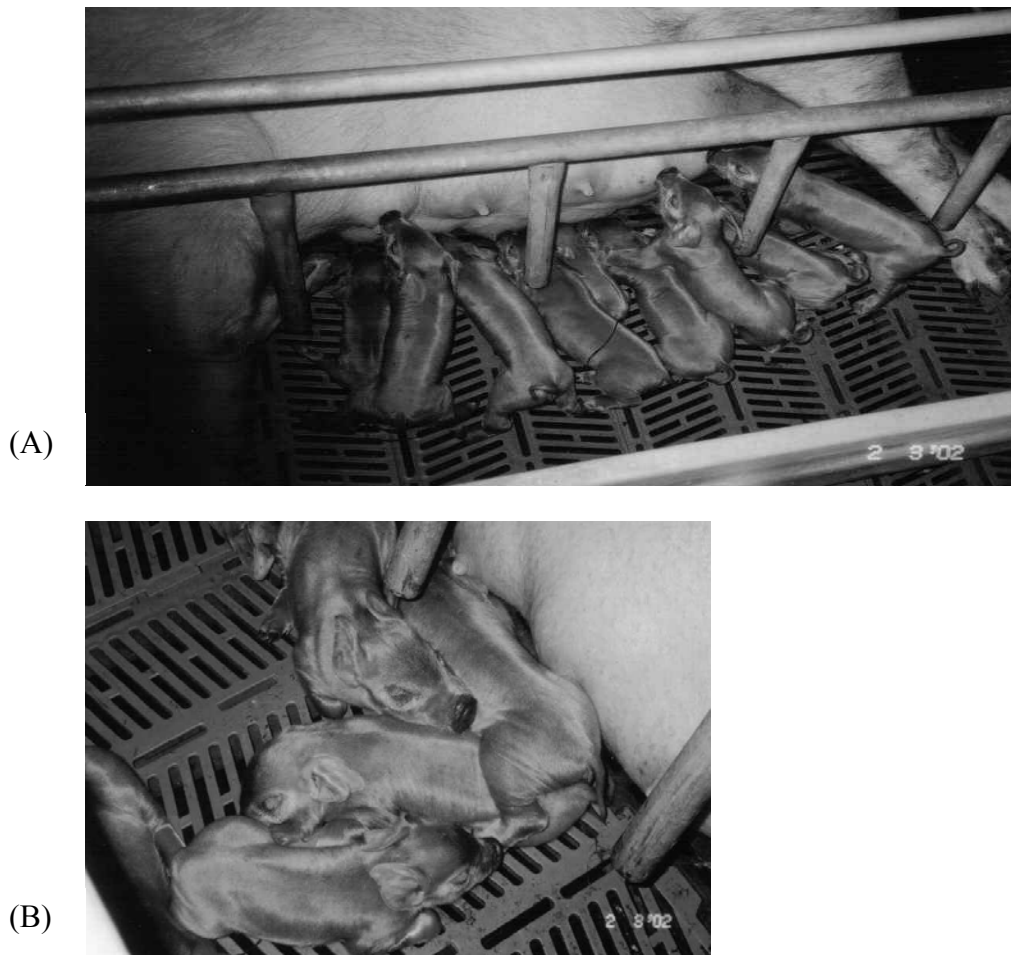


Figure 22. Pictures of the litter of transgenic clones born in February 2002. (A) All nine piglets nursing shortly after birth. The diminutive baby is the piglet in the middle, the fifth on from either end. (B) Closer picture of some of the normal sized piglets.

In addition to the above work with the GT1, another set of experiments incorporating the findings from the enhancer projects mentioned earlier was undertaken. An NLS-bearing protein recognition signal was added to the GT1 in a manner similar to that of the truncated puromycin plasmids and electroporations were performed either with or without thymidine. A summary of the results from these experiments is in Table 13. Unfortunately, no puromycin resistant colonies resulted from any of these electroporations even when the puromycin selection concentration was lowered. In addition, it was seen in this and all other experiments involving thymidine that the cells exposed to thymidine for 24 hours tended to be less robust and did not survive electroporation as well as those cells not treated with thymidine.

Table 13. Summary of GT1/NLS with or without Thymidine

Construct Electroporated	Number of Cells Electroporated	Colonies Surviving Selection
GT1/NLS without Thymidine	26×10^6	0 colonies
GT1/NLS with Thymidine	247.7×10^6	0 colonies

During the course of the above work, three groups reported successful targeting of the $\alpha 1,3$ galactosyltransferase gene (71-73). It was decided to re-develop a construct similar to the one used by Dai et al. (72) (Fig. 16). However, our construct differed in two ways, first it contained most of exon 8 and the 5' end of intron 8 not present in the construct used by Dai et al. and second, we inserted a concatamer of at least three NLS recognition signals at the 3' end of the construct outside the area of homology to GT. Our first goal was to compare the HR efficiencies between the GT1 and GT2 constructs (both containing NLS) therefore nine electroporations of 10×10^6 cells each were performed with the GT2 construct in the absence of thymidine and a smaller experiment of two electroporations (5×10^6 and 2×10^6 cells) was performed with GT2 and thymidine. A summary of results from the GT2 experiments is in Table 14.

Table 14. Summary of GT2/NLS with or without Thymidine

Construct Electroporated	Number of Cells Electroporated	Colonies Surviving Selection
GT2/NLS without Thymidine	90×10^6	672 colonies
GT2/NLS with Thymidine	7×10^6	2 colonies

A total of 674 (~7 colonies per million cells electroporated) colonies were transferred and grown. Of these 356 (~53%) colonies grew well enough to either freeze or extract DNA or both. LR-PCR was performed on all 356 colonies using primer set B/D from Table 9 of which 310 (~87%) gave a PCR result (Fig. 23), however, no colonies gave the expected double band pattern at 7.9kb and 9.6kb representing both the non-targeted and targeted alleles.

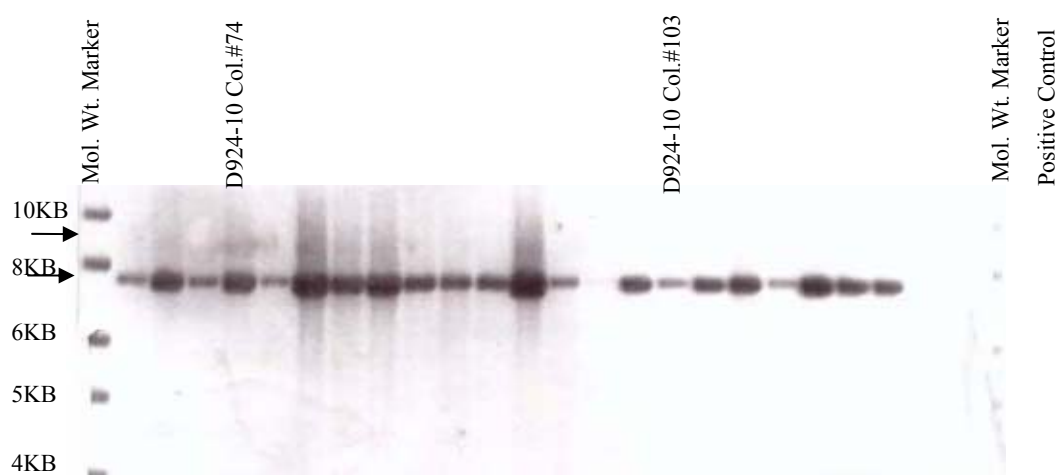


Figure 23. Representative ethidium bromide gel of LR-PCR products using primer set B/D from Table 9. The expected products are bands at 7.9kb (endogenous allele) and at 9.6kb (targeted allele). The lanes marked represent two potential positive colonies. The arrows show the position where the non-targeted and targeted bands should be.

All LR-PCR reactions were separated by electrophoresis, transferred to a membrane, and probed with the IRES probe. From this, twelve “possible positive” clones were identified having a positive band located near the expected size of the targeted LR-PCR product (9.6kb). To further verify these possible positives, their

membranes were stripped and re-probed with the neo probe (Figs. 24 and 25). The results of these two Southern blots showed three colonies that gave a promising band at the expected targeted allele size but also gave unexplainable, additional bands. In addition, DNA from the twelve colonies was amplified using the primer sets, A/E and C/F from Table 9. However, no bands were visible using these primers. As a further test, the PCR products from the C/F reaction were transferred to a membrane and probed with the IRES probe, but no positive signals were found.

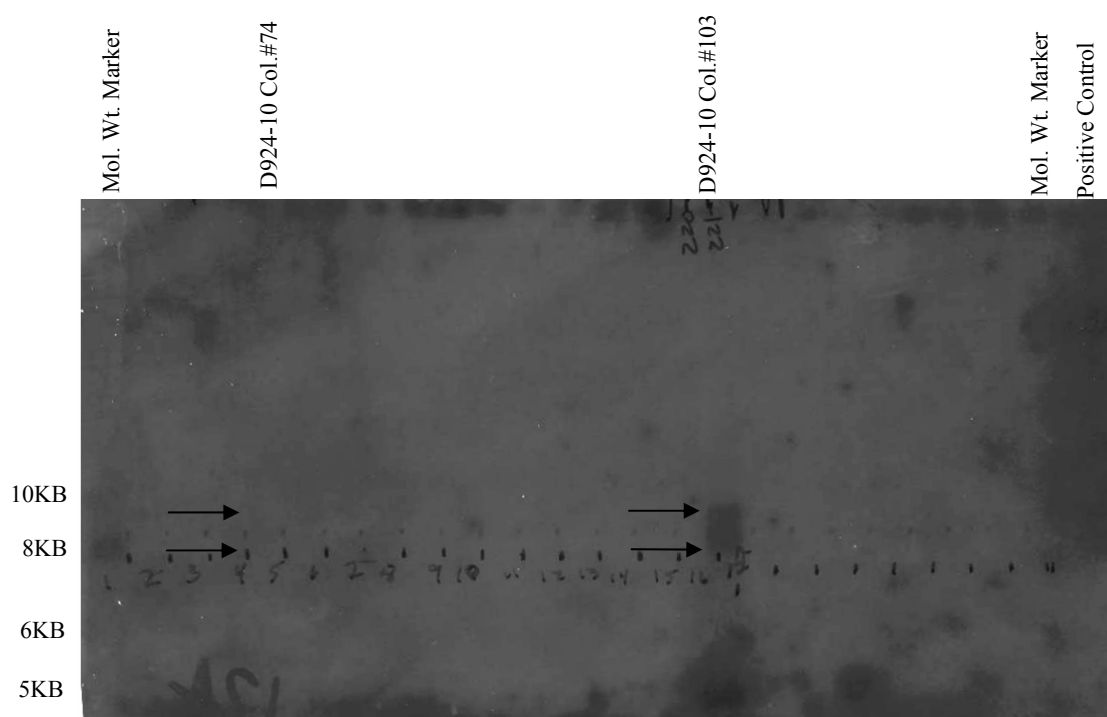


Figure 24. Radiograph of Figure 23 LR-PCR products using primer set B/D from Table 9 probed with the IRES probe. The lanes marked represent the potential positive colonies. The arrows represent the area where the endogenous (7.4kb) and targeted (9.4kb) LR-PCR products should be. Only the targeted band should react with the IRES probe used.

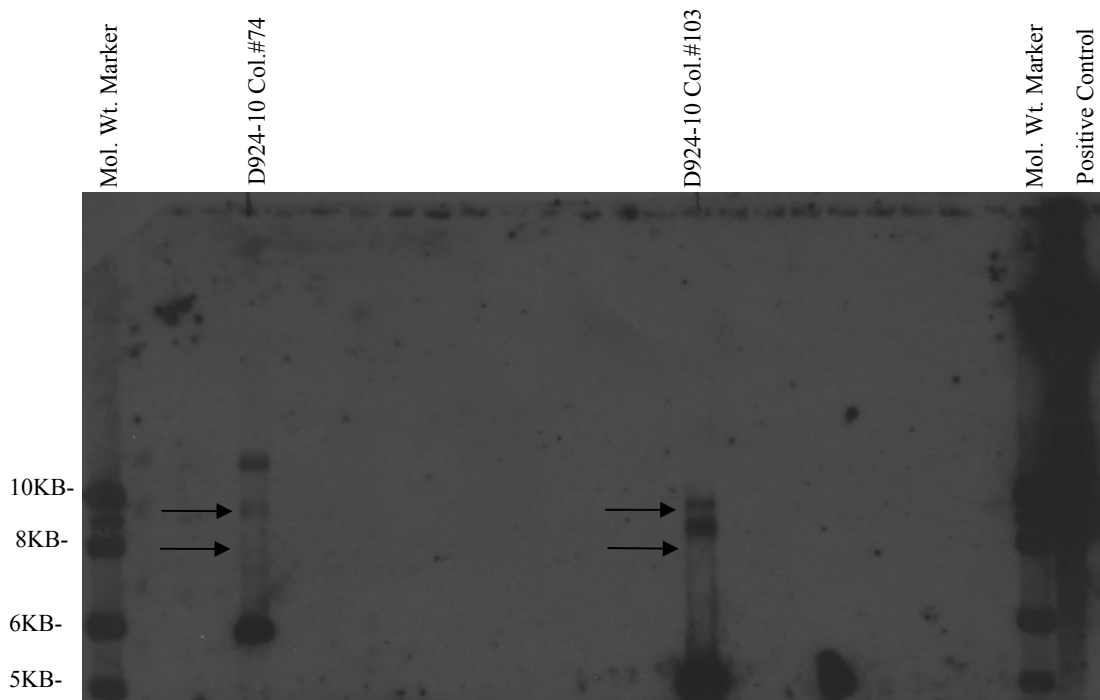


Figure 25. Radiograph of Figure 23 LR-PCR products using primer set B/D from Table 9 probed with the neo probe. The lanes marked represent the potential positive colonies. The arrows represent the area where the endogenous (7.4kb) and targeted (9.4kb) LR-PCR products should be. Only the targeted band should react with the neo probe used.

DISCUSSION

The three groups (71-73) that have reported successful targeting of the GT gene utilized different transfection, construct, or selection mechanisms resulting in outcomes of efficiency that are difficult to compare. Harrison et al. (73) attempted targeting first with a GFP fusion protein directly upstream of a pgk puromycin gene which was inserted into and disrupted exon 9. They chose a lipofection based method to introduce

the construct and passed the cells to lower density plates 24 hours after transfection. Of the 1400 puro resistant colonies, zero were found to produce enough GFP product to be measured by FACS and concluded that perhaps the GT promoter was too weak to drive sufficient amounts of GFP. Using LR-PCR one putative positive was identified giving a 0.07% targeting efficiency (1 colony divided by 1400 colonies multiplied by 100). Their second attempt at targeting utilized a neo fusion promoter trap construct (without an IRES) inserted/disrupting exon 4. They again chose lipofection and passed cells to lower density 24 hours after transfection. In this experiment, however, they divided the cells into two groups, low O₂ and ambient O₂ atmosphere. The low O₂ group seemed to do better in that of the 225 G418 resistant colonies found, six were positive targeting events giving a 2.7% targeting efficiency. The ambient O₂ group resulted in 170 colonies of which three were positive giving a 1.8% targeting rate. Overall, 395 colonies were analyzed and nine were found to be positive giving an overall targeting rate of 2.3%.

Lai et al. (71), while basically interrupting exon 9 in the same way as Dai et al. (72) (an IRES fused to a promoterless neomycin resistant gene), used a construct which was considerably longer and had more homology to the GT gene (21kb versus 8.6kb). They chose to transfect only the GT DNA minus the vector backbone into the cells by electroporation and passage to lower density after 48 hours. They also chose to use a cell line from a highly inbred strain of miniature pigs and not the out-bred breeds of pigs used for all other GT work. Given the data reported, 159 G418 resistant colonies were

analyzed and eight were determined positive giving a targeting efficiency of 5%, the highest of all groups.

Dai et al. (72) used the IRES fused to a promoterless neomycin resistant gene inserted/disrupting exon 9. Electroporation of this construct with the vector backbone followed by passage to lower density 48 hours later resulted in 1105 G418 resistant colonies of which 30 probable (17 proven) colonies were targeted. This gave a 2.7% targeting efficiency.

In the work presented here no successful targeting events were found. Using the GT1 vector without the NLS signal resulted in 37 colonies analyzed from electroporation experiments and 31 colonies analyzed from lipofection experiments. The GT1-NLS construct gave no colonies after electroporation either with or without thymidine. Electroporation of the GT2 construct resulted in the analysis of 356 colonies. In addition to determining targeting efficiency, it is important to normalize the data to the number of cells transfected and, by extrapolation, how many colonies would result per million cells transfected. A summary of all the experiments mentioned above is given in Table 15.

Table 15. Summary of published GT targeting statistics

Group Targeting	# Cells Transfected	# Colonies Survive To Analysis	# Colonies Per Million Transfected	# Positive Colonies	% Efficiency
Harrison (Puro)*	11.5x10 ⁶	1400	122	1	0.07%
Harrison (Neo)*	0.252x10 ⁶	395	1567	9	2.3%
Lai**	80x10 ⁶	159	2	8	5%
Dai**	14x10 ⁶	1105	80	30	2.7%
GT1 EP	1005x10 ⁶	37	0.04	0	0%
GT1 Lipo	68x10 ⁶	31	0.5	0	0%
GT1-NLS	273x10 ⁶	0	0	0	0%
GT2-NLS	97x10 ⁶	356	4	0	0%

* Passage of cells 24 hours after transfection

** Passage of cells 48 hours after transfection

Because of the differences between experiments, the only realistic comparison is between Dai et al. and the GT2-NLS. While it is sometimes unwise to compare experiments from different laboratories and because Dai passed their cells after transfection, therefore possibly skewing the numbers, one can make some tentative comparisons between the two sets of experiments. There was a 20-fold decrease in the number of colonies per million cells transfected when an NLS was present on the construct. The presence of an NLS could be responsible for lowering the amounts of

random integrations and therefore lessening the number of colonies surviving selection. It should also, theoretically, enrich for HR events however we were unable to identify any positively targeted colonies. Given the targeting efficiency of 2.7% found by Dai, our experiment should have resulted in 8 of the 356 colonies analyzed being positively targeted. Taking into account the passage of the cells 48 hours (and thus two cell cycles/replications of the cells) before selection was added, we could assume at least one if not two doublings of the cell population giving a 2 to 4 times increase in the targeting efficiency found by Dai et al. (72). This would lower the value of the expected colonies to be found in the 356 colonies isolated from the GT2-NLS experiment to 2 to 4 colonies. This value reflects what would be expected without the added enrichment of the NLS. We would expect a higher number of positive colonies with NLS enrichment. Because we were unable to identify any positive colonies, it is difficult to conclude if the differences between the two laboratories were the cause of the differences in targeting efficiencies or if a larger number of colonies are need to identify a positive target.

A comparison of colony survival to analysis and the number of colonies per million cells transfected between the GT1 experiments when looked at from promoter trap—lipofection vs. electroporation and polyA trap—lipofection vs. electroporation shows a 100% survival rate of all lipofected colonies while only 22% promoter trap and 52% polyA trap electroporated colonies survived to analysis. In addition, lipofection was more favorable to growth with 0.5 and 0.25 colonies per million cells for promoter trap and polyA trap compared to 0.02 and 0.06 colonies in electroporation of the constructs. The differences in the numbers of colonies surviving lipofection can be

explained by the more favorable nature of this form of transfection over electroporation. Lipofection involves the transfer of DNA through the cell membrane via endocytosis of a DNA-lipid complex (186) while electroporation relies on an electric field to create transient holes in the cell membrane (187). While too few cells were subjected to lipofection to draw any definite conclusions, sufficient cell numbers were electroporated to show the predicted three-fold increase in colonies expected in the polyA trap compared to promoter trap constructs. The presence of an NLS in the GT1-NLS construct dramatically reduced the number of cells surviving selection as compared to the GT1 promoter trap electroporations. While the number of cells electroporated was sufficient to expect colonies, the lack of colonies with the GT1-NLS with or without thymidine further supports the trend seen with the GT2-NLS plasmid (as compared to Dai et al. (72) results) in the NLS signal's ability to reducing the number of colonies.

The presence of an IRES greatly increases the number of colonies due to its ability to allow for cap-independent translation of the selectable marker located directly downstream. In essence, the researcher loses some of the power associated with a promoter trap when an IRES is used in the construct. If the targeting construct incorporates into any actively transcribed gene, the IRES will allow for translation regardless of the position of the start codon of the selectable marker. The addition of the IRES is necessary in some cases when the endogenous promoter of the gene of interest is weak and can not drive enough selectable marker product to allow for survival of the correctly targeted cells. Because Harrison et al. (73) found the GT promoter activity insufficient to drive GFP expression, the addition of an IRES in the targeting construct is

probably warranted. However, Harrison et al. (73) successfully used a fusion neomycin resistant gene without an IRES but their construct utilized a different area of the GT gene and therefore may lead to different targeting efficiencies. Further support to a promoterless neomycin resistant gene's ability to protect the cell was given by Hanson and Sedivy (188) who achieved a 4% targeting efficiency in an immortal rat fibroblast cell line using a relatively weak *c-myc* gene promoter trap construct. The *c-myc* promoter activity was sufficient to drive neomycin resistance despite the presence of 33 times more G418 than normally used. If the GT promoter is a weak expresser, the lack of an IRES in the GT1 (promoterless puromycin) constructs could hinder the ability of the puromycin resistant gene to elicit protection making it impossible to identify any positively targeted colonies.

Overall, these results suggest that the use of a promoterless puromycin resistant gene may not be the best choice for use in targeting the GT gene. The choice of puromycin instead of neomycin/G418 for selection was made mainly because of puromycin's ability to kill cells more quickly and therefore lead to less background in culture and lessen the amounts of mixed (both transgenic and non-transgenic) populations of cells in the surviving colonies. However, puromycin is probably too powerful a selection molecule to use when the weaker GT promoter drives the resistant gene and therefore targeted colonies may have died during selection despite using the lowest possible concentration of puromycin. In order to use puromycin for selection with low active promoters a new, more efficient puromycin resistant gene needs to be developed. The use of a promoterless neomycin resistant gene without an IRES has

been successful and fewer colonies arise because of the lack of the IRES. However, because of the chance of either the fusion protein not functioning properly or the endogenous promoter not being strong enough, the use of an IRES in the targeting construct is warranted at least in the preliminary experiments.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The ability to precisely modify the genome of livestock species will have profound effects in both the fields of agriculture and human medicine. The successful attempts made thus far are very encouraging. The current protocols used are not efficient enough to make targeting in livestock a routine technique. Because of the large expense of raising, maintaining, and breeding livestock, protocols that increase the efficiency of targeting are of the utmost importance. The aim of these experiments was to develop an efficient HR system for the pig.

HR in somatic cells is a complex and poorly understood phenomenon. The first study attempted to manipulate the somewhat simpler NHEJ pathway in order to indirectly up-regulate HR. Three molecules were chosen to study; Mre11, Ku70/80, and p53. Unfortunately, manipulation of the Mre11 and the Ku proteins did not increase HR between the two truncated plasmids. Mutation of the p53 protein did show significant improvement of HR, however, tight control must be exerted on the transient nature of the p53 gene due to the tendency of mutated p53 cells to become aneuploid thus limiting the application of the cells.

Another major obstacle to overcome in the use of normal somatic fibroblasts in targeting experiments is their finite lifespan. Human fibroblasts have been made to bypass senescence by the addition of hTERT. Transformation of PFFs with the same hTERT did not extend lifespan. Also, cells high in telomerase activity have higher rates of HR. To test whether telomerase expression, in itself, caused this increase in HR,

PFFs expressing hTERT were compared to control PFFs not expressing hTERT in their ability to recombine two truncated plasmids. There was no increase found, however, given the fact that hTERT did not increase the lifespan of PFFs it is unclear whether hTERT functions in the same manner in PFFs as porcine telomerase. Once a porcine TERT is isolated and shown to function correctly, these experiments should be repeated to elucidate an important aspect of HR. As an added benefit, extension of the lifespan of PFFs while maintaining normal karyotypes, as shown with hTERT expressing human fibroblasts, will greatly facilitate the selection process possibly making two rounds of selection possible. This will dramatically decrease the time and expense of making a homozygous knockout animal.

As another way to improve HR, the time of the cell cycle as well as efficient DNA nuclear delivery was studied. Thymidine treatment was used to synchronize the cells in late S phase and an NLS-bearing protein recognition signal was added to the two truncated plasmids. Improvement of HR resulted in each situation with a slight additive affect using both. HR is most active in the late S phase of the cell cycle; therefore, synchronizing the cells allows for more cells to be receptive to repairing the DSBs in this fashion over that of NHEJ. Thymidine, however, has the potential to harm the cells as seen by the poor quality of the cells after treatment and the lower survival after electroporation. Studies determining other, less detrimental, late S phase synchronizing agents should enhance the efficiency of HR. Other DNA delivery mechanisms such as lipofection could also help to alleviate some of the harmful affects of thymidine treatment. The expedient delivery of the plasmid to the nucleus lessens the time in the

cytoplasm where it can be rapidly degraded by various nucleases, as well as extending the time of plasmid exposure to the HR machinery in the nucleus also increases the rates of HR. The NLS signal used in the experiments functioned well. The use of this or other more specific (i.e. protective, cell cycle specific, etc.) NLS recognition signals should help to further the gene targeting efficiency.

Finally, because of the discrepancies sometimes seen when results from plasmid to plasmid (extra-chromosomal) recombination experiments are applied to targeting (inter-chromosomal) protocols, the enhancer protocols were applied to the targeting of the GT locus. Extensive experiments using a promoterless puromycin resistant gene were undertaken. Comparatively few colonies survived to analysis and none were deemed positive. One promising colony was used for cloning and resulted in the birth of eight healthy transgenic piglets. Puromycin may be too powerful a selection agent to use when a relatively weak promoter such as the GT is used to drive the expression of the currently available resistant gene. Development of a more potent puromycin resistant gene could facilitate the use of this selection agent in future targeting projects.

The use of an NLS signal and thymidine synchronization greatly reduced the number of colonies surviving selection over that of a similar construct used by Dai (72). Three of these colonies were promising, but due to the finite lifespan of the fibroblasts, sufficient growth to isolate enough DNA for a genomic Southern blot was impossible. Therefore, a definitive answer to whether a gene targeting event took place is not possible. Cloning of these colonies to produce fetuses or live piglets would enable the collection of tissue on which a genomic Southern blot could be performed. In addition,

more transfections need to be performed to determine a definitive answer as to whether the enhancers do indeed work for gene targeting as they do with the plasmid to plasmid systems. The results could also be locus dependent. In related experiments the use of the NLS and thymidine synchronization resulted in increased rates of HR at the HPRT locus in bovine fetal fibroblasts (Mir and Piedrahita unpublished observations).

The ability to achieve gene targeting using livestock somatic cells is a powerful tool. Studies on the basic mechanisms involved in HR and NHEJ as well as the development of protocols to manipulate other aspects that indirectly affect the rates of HR (i.e. telomerase, cell cycle, and DNA delivery) will benefit all aspects of biological science. More efficient protocols will allow for targeting techniques to be used by laboratories that would normally not undertake such studies due to its current inherent difficulties.

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VITA

Gretchen Marie Zaunbrecher was born December 4, 1970 in Crowley, Louisiana to Steve and Rose Marie Zaunbrecher. Gretchen was raised with her older sister, Sandy, and her older brother, Quentin, in Roberts Cove, Louisiana, a small German settlement in the middle of Cajun Louisiana. Gretchen graduated from Notre Dame High School in 1988 and obtained her Bachelor of Science degree in animal science from the University of Southwestern Louisiana in 1992. She then moved to Lubbock, Texas to attend Texas Tech University where she received her Master of Science degree in reproductive physiology, under the direction of Dr. Scott Whisnant. She received her degree in May 1996 and stayed in Lubbock to work as a laboratory technician at the Southwest Cancer Center for a year and a half. She then moved to College Station, Texas and enrolled at Texas A&M University where she pursued a Doctor of Philosophy degree in the Department of Veterinary Anatomy and Public Health under the guidance of Dr. Jorge Piedrahita in the area of transgenics in pigs. She began her work in January 1998 and graduated in May 2004.

Permanent address: 5373 Roberts Cove Road, Rayne, LA 70578